The Role Of Intracellular Signalling Pathways In Conferring Resistance To Endocrine Therapies In Breast Cancer

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"Success consists of going from failure to failure
without loss of enthusiasm"

Winston Churchill

In accordance with the regulation of the University, I declare that this thesis has been completed by myself entirely, and that the work presented here is my own, except where acknowledgment had been made in the text.

Vera Cerqueira
September 2009

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Abstract

Breast cancer is the most prevalent form of cancer in women and accounts for 519,000 annual deaths (WHO Statistics). It has long been established that oestrogen (E_2) stimulates tumour growth of oestrogen receptor (ER) positive breast cancer and is involved in the pathogenesis of the disease. Consequently, therapeutic approaches targeting the ER were developed. The use of endocrine therapy is an integral component in treating breast cancer however resistance to such drugs is a major limitation. Unfortunately, even initially responding tumours eventually develop resistance - *acquired resistance*.

The aim of this study was to determine which intracellular pathways may be important in conferring acquired endocrine resistance. In order to do so, a three-stage MCF-7 cell model emulating the clinical development of acquired endocrine was used. MCF-7/LCC1 (LCC1) and MCF-7/LCC9 (LCC9) cells lines were derived from the oestrogen dependent and anti-oestrogen sensitive MCF-7 cell line. LCC1 cells remain responsive to endocrine therapies but their growth is not dependent on oestrogenic stimulus. LCC9 cells, on the other hand are fully resistant to endocrine therapies and completely oestrogen independent.

A number of different cell membrane receptors and intracellular pathways have been implicated in endocrine resistance including HER receptor family, PI3K/Akt & MEK/ERK pathways. These pathways are of particular interest since they are able to activate ER in the absence of oestrogenic stimulus. It is likely that several pathways may be important in conferring resistance to endocrine therapies therefore the experiments in this study focussed on the transcriptional regulation of HER receptors, the activation of the Akt pathway and its implication to basic cellular processes.

Following E_2 treatment (48h), HER2/3/4 mRNA and protein levels were reduced in MCF-7 and LCC1 but not in the endocrine-resistant LCC9 cell line as measured by QRT-PCR and Western blotting. The anti-estrogen fulvestrant (ICI 182,780) reversed the E_2 modulation. A previous study has shown that ER and the *HER2* promoter compete for limiting amounts of SRC-1 in oestrogen-responsive ZR-75-1 cells, causing HER2 repression after E_2 stimulation (Newman et al.,Oncogene, 19, 490-7, 2000). ER RNAi abolished E_2 repression of HER2 in MCF-7 and LCC1 cells. Furthermore, LCC9 cells have reduced SRC-1 recruitment to ER (assessed by ChIP) allowing SRC-1 to bind to the *HER2*

promoter. SRC-1 RNAi reduced HER2 transcription in MCF7 cells in a manner similar to E_2 whilst it did not restore E_2 repression in LCC9 suggesting that the latter cells have alternative mechanisms regulating *HER2* transcription. RNAis against the other two p160 co-activators TIF2 and AIB1 did not restore E_2 mediated HER2 repression in LCC9 cells. The importance of redundancy between p160 co-activators was also determined by performing double knockouts. SRC-1/TIF2 and TIF2/AIB1 double siRNAs had little effect on HER2 mRNA levels however SRC-1/AIB1 siRNA restored oestrogen mediated downregulation of HER2 transcription in LCC9 cells. This data indicates that SRC-1 and AIB1 co-activators play a role in the transcriptional regulation of HER receptor particularly in MCF-7 and LCC1 cells. The regulation of this transcriptional mechanism is altered in resistant LCC9 cells but, as evidenced by the double knockouts, p160 co-activators are still able to affect HER expression in these cells. This mechanism was further studied in primary breast cancer tumour material.

The importance of the Akt pathway in this cell line model was also investigated as phospho-Akt levels are elevated in LCC1 and LCC9 cells. This in turn was shown to activate mTOR and ER (Ser167 residue phosphorylation) thereby contributing to increased growth and ligand independent activation of the oestrogen receptor respectively. Activation of PI3K and PTEN is unchanged in LCC1 and LCC9 cells suggesting that these proteins are not responsible for elevated Akt phosphorylation. In contrast, these cells do express higher levels of phospho-IGFR due to the high availability of receptor ligands (IGFI & IGFII). This is likely to be, at least partially, responsible for the elevated Akt activation. Moreover, the role of Akt isoforms was also determined as they are known to have different functions. The levels of Akt 2 phosphorylation are higher in endocrine resistant cell lines in comparison to parental MCF-7 cells. Interestingly, the Akt 3 phosphorylation is present in all cell lines whilst Akt 1 phosphorylation is minimal. Nevertheless, Akt RNAi studies reveal that Akt 1 and 2 siRNA dramatically reduce growth in MCF-7, LCC1 and LCC9 cells. These results suggest that Akt 2 phosphorylation may play a part in conferring endocrine resistance but the other isoforms are also important for normal cellular growth.

The cell cycle profiles of LCC1 and LCC9 are very similar to MCF-7. Similarly, migration levels are unchanged in endocrine resistant cell lines. However, in the presence of anti-oestrogenic drugs, apoptosis in LCC1 and LCC9 cells in reduced in comparison to the

parental MCF-7 cell line. Furthermore, LCC1 and LCC9 cells have higher invasion rates. The deregulation of HER receptor expression and elevated Akt activation may together confer survival advantage in LCC1 and LCC9 cells whilst also increasing their invading potential.

Abbreviations

ºC	Degrees centrigade
CARM1	Co-activator associated arginine methyltransferase
CDK2	Cyclin A
DMSO	Di-methyl sulphoxide
DCIS	Ductal carcinoma *in situ*
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ECM	Extracellular matrix
ERE	Oestrogen responsive element
ERK	Extracellular regulated kinase
h	Hours
HAT	Histone acetyltransferase
HCl	Hydrochloric acid
HRT	Hormone replacement therapy
IGFR	Insulin-like growth factor receptor
LCIS	Lobular carcinoma *in situ*
mg	Milligram(s)
ml	Milliliter(s)
MAPK	Mitogen activated protein kinase
MEK	Mitogen extracellular kinase
min	Minute(s)
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCOR	Nuclear receptor corepressor
PBS	Phosphate buffered saline
PCAF	p300/CBP associated factor
PI3K	Phosphotidylinositol-3 kinase
PIP	Phosphatidylinositol phosphate

PKB	Protein kinase B
PR	Progesterone receptor
PTEN	Phosphatase & tensin homologue
SERM	Selective oestrogen receptor modulator
SH2	SRC homology 2
SWI/SNF	Switch/sucrose non-fermenting
SMRT	Silencing mediator for retinoid and thyroid receptors
TBS	Tris buffered saline
TGF	Transforming growth factor
TR	Thyroid receptor
V	Volts

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Chapter 1

Introduction

1.1 Breast Biology

Breast cancer is the most frequent type of cancer affecting women worldwide and the second leading cause of cancer deaths in females after lung cancer. The development of endocrine resistance in breast cancer is one of the major challenges in treatment of this condition.

1.1.1 Normal Breast

The development of the mammary gland begins in the womb and its structure is similar in females and males at birth. The breast tissue changes progressively throughout childhood but becomes strikingly different at puberty in women. The development of the mammary glad at puberty is dependent on the presence of the hormones oestrogen and progesterone produced by the ovaries (Russo *et al*, 2000). Later in life, the breasts undergo a relentless cycle of growth followed by involution (mediated by apoptosis) regulated by the menstrual cycles or by the pregnancy/lactation cycles (Ali *et al*, 2002).

The mammary gland is comprised of a simple network of ducts originating during intrauterine life which later form lobules (each mammary gland may have up to 20 lobules) (Figure 1.1). At puberty, the ducts begin to grow and divide thereby increasing ductal branching and forming terminal end-buds. These structures will, in pregnancy, give rise to the alveolar buds that are responsible for milk synthesis. Each duct is made of two cell layers: an epithelial cell layer (milk production) surrounded by the contractile myoepithelial cells (Russo *et al*, 2000; Ali *et al*, 2002).

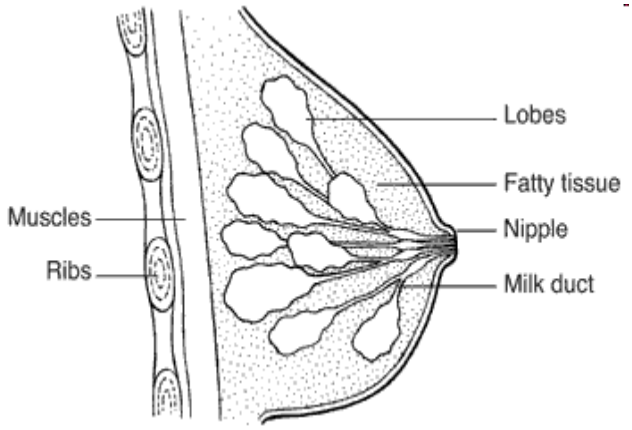


Figure 1.1 Anatomy of normal mammary gland. These are composed of fatty and connective tissue surrounding the lobes where the milk is produced. A network of ducts connects the lobes to the nipple (Reproduced from www.cancerbackup.org.uk).

1.1.2 Breast Cancer

1.1.2.1 Incidence & Survival

Breast cancer is detected in approximately 1 million women around the world each year. In the UK, 45,000 people are diagnosed with this type of cancer each year, 300 of which are in men (Cancer Research UK statistics). In the United States, around 250,000 new cases are diagnosed annually (American Cancer Society, 2007). After non-melanoma skin cancer, breast cancer is the most common cancer in the world and is the second leading cause of cancer death after lung cancer (Stewart *et al*, 2004). There is a lifetime risk of developing breast cancer of 1 in 9 which increases with age (Ali *et al*, 2002). Most women are diagnosed with breast cancer after menopause but there are a significant number of cases in women under 50 years of age perhaps due to familial and genetic predispositions (American Cancer Society, Cancer Facts & Figures, 2006). Breast cancer is responsible for an average of 40,000 and 12,000 deaths in the US and the UK respectively. Nevertheless, the death rates for this disease have actually reduced since 1990, particularly in younger women which is probably a result of increased awareness, early detection and improved therapeutic approaches (American Cancer Society, Cancer Facts & Figures, 2006).

1.1.2.2 Epidemiology

Breast cancer may be divided into a number of different categories based on the location and characteristics of the cancerous cells. Lobular carcinoma in situ (LCIS) refers to tumours confined to the breast lobules, the milk producing glands. Ductal carcinomas in situ (DCIS), on the other hand, are restricted to the lobule ducts. Both these types of breast cancer remain in the site of origin (hence the term in situ) and have not spread to the neighbouring breast tissue (Weigelt *et al*, 2009). DCIS morphologically resembles the ductal types of invasive breast cancers whilst LCIS are similar to invasive lobular breast cancer suggesting they may be precursors for more invasive breast cancer types (Buerger *et al*, 2000). Nevertheless, there is evidence to suggest that some DCIS cases are associated with invasive lobular carcinomas and that a small proportion of LCIS may give rise to invasive carcinomas of the ductal subtype (Millikan *et al*, 1995).

Approximately 80% of diagnosed breast cancer cases are invasive ductal carcinomas making it the most common type of breast cancer followed by invasive lobular carcinomas. There are other more rare types of breast cancer such as inflammatory breast cancer (1-2%) and Paget's disease (1-2%). Inflammatory breast cancer is characterised by inflamed breast

tissue due to the blockage of lymph ducts (Molckovsky *et al*, 2009). The symptoms associated with Paget's disease include a red rash around the nipple and areola which if left may ulcerate. This condition is often mistaken for eczema and its causes remain unknown. Approximately 90% of women suffering from Paget's disease have an underlying breast cancer usually DCIS or invasive ductal carcinoma (Dalberg *et al*, 2008).

1.1.2.3 Stages of Breast Cancer

Cancer staging is a helpful tool for both doctors and patients as it provides a means of placing the disease into context (Table 1.1). The staging system takes into account a number of tumour properties such as the size of the tumour, its aggressiveness and its invading potential. This mechanism enables patients a more in depth understanding of their condition, their prognosis and the appropriate therapeutic approaches. Furthermore, this method provides the health professionals involved in cancer treatment with a useful manner in which to communicate (Jeruss *et al*, 2008).

Stages		**Description**
Stage 0		Non-invasive breast cancer such as DCIS and LCIS Cancer has not spread from site of origin
Stage I		Invasive cancers; lymph nodes not affected Tumours < 2cm diameter
Stage II	**A**	Cancer cells only present in axillary lymph nodes OR <2cm tumour and cancer cells found in lymph nodes OR 2-5 cm tumour; no cancer cells found in axillary lymph nodes
	B	2-5cm tumour; cancer cells present in axillary lymph nodes OR tumours >5cm diameter; no cancer cells found in lymph nodes
Stage III	**A**	Tumours over 5cm diameter; cancer cells found in lymph nodes; characterised by clumping of lymph nodes and surrounding tissue
	B	Tumours may be any size; tumour has spread to skin and chest wall; cancer cells present in lymph nodes; inflammatory breast cancers fall into this category
	C	Tumours may be any size; cancer cells have spread to lymph nodes in: armpit and under breast bone OR above/below collarbone
Stage IV		Tumours has spread to other parts of the body; Metastatic cancer

Table 1.1 Stages of breast cancer disease.

1.1.2.4 Role of Oestrogen in Breast Cancer

Oestrogen has been implicated in breast cancer since 1896 when George Beatson showed that performing an oophorectomy suppressed breast cancer growth. As previously described, breast development at puberty is dependent on hormones produced by the ovaries such as oestrogen. Despite its crucial role in regulating mammary growth, high levels of oestrogen appear to be associated with an increase in breast cancer risk (Clemons *et al*, 2001).The exact mechanism by which oestrogen increases the risk of breast cancer is still to be fully determined (Yager *et al*, 2006).

The exposure to endogenous and exogenous oestrogens during a woman's lifetime seems to be a critical factor in breast carcinogenesis. Oral contraception and hormone-replacement therapies have both been shown to correlate with an increase in breast cancer risk (Collaborative Group on Hormonal Factors in Breast Cancer, 1996 & 1997). This will be further discussed later in this chapter (Section 1.1.2.5. (ii)). There are other well-recognised factors that should be taken into account referring to certain reproductive characteristics such as age at menarche, age at first birth, number of children and breastfeeding. The early onset of menarche and late menopause are linked to increased breast cancer risk probably due to the longer exposure to oestrogen (Clamp *et al*, 2002). Early first pregnancies, lactation and parity have all been shown to have a protective effect against breast cancer which may be due to the differentiation process of the terminal duct lobules or to the various hormones present at lactation (Albrektsen *et al*, 1999; Clamp *et al*, 2002).

1.1.2.5 Risk Factors in Breast Cancer

(i) Age

Age is one of the major contributors in breast cancer as over 80% of cases occur in women over the age of 50 (Breakthrough Breast Cancer Statistics). However, the risk of developing breast cancer reduces dramatically following menopause probably due to the reduction of circulating oestrogen (Eerola *et al*, 2002). At menopause the ovaries cease to produce oestrogen, therefore this hormone is no longer available to promote growth of breast cancer tumours.

(ii) Hormone Replacement Therapy & Oral Contraceptives

Hormone replacement therapies (HRT) are routinely used in menopausal women to reduce the effects of diminished levels of circulating oestrogen. These treatments improve patients' quality of life and also prevent conditions such as osteoporosis, colon cancer and dementia (Bluming *et al*, 2009). Despite the advantages of HRT, a number of studies have highlighted that they may also increase the risk of diseases such as cardiovascular disease, breast cancer and stroke (Nelson *et al*, 2002). There are conflicting reports regarding the risk of breast cancer whilst on HRT. A few studies have not found any association between the two (Grady *et al*, 1992; Hemminki *et al*, 1997; Steinberg *et al*, 1994) whilst others, including the study from the Women's Health Initiative published in 2002, claim that HRT use significantly increases the risk of breast cancer (WHI report, 2002; Sillero-Arenas *et al*, 1992; Colditz *et al*, 1993). In spite of the contradicting results it is becoming apparent that women using oestrogen in combination with progesterone are at higher risk of developing breast cancer after 5 years treatment, more so than patients on oestrogen only treatment (Burger *et al*, 2003). Furthermore, reports also indicate an increase in risk with long term treatment (Nelson *et al*, 2002).

Similarly to HRT use, there are conflicting results regarding the link between the oral contraceptive pill and breast cancer. A recent study suggested there was no association between breast cancer and the use of the oral contraceptive pill after examining data from over 4,500 women (Barnett *et al*, 2008). On the other hand, in 1996 data compiled from 54 published reports suggested that women currently using combined oral contraceptives were found to be at higher risk of developing breast cancer. Furthermore, the risk increased after 10 years of treatment (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Travis *et al*, 2003).

(iii) Lifestyle

There are a number of factors that have been linked to breast cancer, either as protective or as potential contributors to the disease. A number of studies suggest physical activity may have a role in preventing breast cancer, especially in post-menopausal women (Pan *et al*, 2009). Obesity in post-menopausal women, on the other hand, has been strongly linked to increased risk (2 fold increase) of breast cancer (Dignam *et al*, 2006). There is conflicting

evidence regarding the role of diet with a suggested positive influence of fruits and high fibre foods compared to red meat and fatty dairy products (Soerjomataram *et al*, 2009).

A few studies have also shown an association between alcohol consumption and increase in breast cancer risk (Smith-Warner *et al*, 1998; Hamajima *et al*, 2002). The mechanism behind this relationship is not fully understood. Certain studies propose alcohol may interfere with folate or oestrogen metabolism whilst other hypotheses suggest carcinogenic metabolites might be important (Chen *et al*, 2005). Interestingly, cohort studies show a dose-response relationship between the amounts of alcohol consumed and breast cancer risk (Zhang *et al*, 2007).

The relationship between smoking and breast cancer is still undetermined due to a variety of discrepant studies (Palmer *et al*, 1993). Mutations of the p53 gene and smoking-specific DNA adducts have been detected in breast tissue suggesting an association (Rundle *et al*, 2002; Conway *et al*, 2002). On the other hand, other studies have failed to detect any relationship between the two. The conflicting results probably reflect variation with the populations in each study (Palmer *et al*, 1993).

1.1.2.6 Genetic Factors

Most breast cancers are said to be sporadic as they occur in women with no family history. However, 20 to 30% of cases are linked to family history and a number of genes have been implicated in this mechanism (Edlich *et al*, 2005).

(i) BRCA 1 & BRCA 2

Most familial breast cancer cases are a result of germ line mutations in *BRCA1* (chromosome 17) or *BRCA2* genes (chromosome 13). These susceptibility genes were first identified in the 1990s and have since been the focus of a number of studies (Miki *et al*, 1994; Wooster *et al*, 1995). In women carrying such mutations, the lifetime risk of breast cancer by the age of 70 is estimated to be 80% (Krainer *et al*, 1997). The *BRCA1* protein is involved in regulating cellular proliferation ensuring the fidelity of DNA replication. *BRCA2* is also involved in maintaining genome integrity due to its role in DNA repair (Sakorafas *et al*, 2000). Both genes are large and over 250 and 100 disease-causing mutations have been described in *BRCA1* and *BRCA2* genes, respectively (Blackwood *et al*, 1998; Couch et al, 1996). These mutations are not confined to particular regions of the gene however large family-linked studies have helped determine that few of these mutations are

recurrent making mutation screening more accessible (Couch *et al*, 1996). *BRCA1* and *BRCA2* are tumour suppressor genes since mutations reducing protein levels or rendering them not functional are associated with tumour formation (Stratton *et al* 1996).

(ii) p53

The p53 protein plays a part in regulating cell cycle checkpoint and is important in the response to DNA damage by inducing cell cycle arrest, DNA repair or apoptosis. In the absence of a functional p53 protein, cells are able to bypass apoptosis thereby replicating damaged DNA (Sakorafas *et al*, 1994). The *p53* gene is found on chromosome 17 and is mutated in about 50% of all human cancer cases. It is known to be a tumour suppressor gene frequently inactivated by point mutations, particularly missense mutations (Sakorafas *et al*, 1994; Cho *et al*, 1994). Around 30% of invasive breast cancers are positive for p53 mutations and these also seem to be associated with poorer prognosis of women with early-stage breast cancer (de Cremoux *et al*, 1999; Linjawi *et al*, 2004).

(iii) PTEN

Mutations of the *PTEN* tumour suppressor gene have also been implicated in breast cancer as they often occur in patients suffering from Cowden Syndrome (80% of cases) (Lynch *et al*, 1997). This rare hereditary condition is characterized by an increase risk of developing certain cancers and by the formation of tumour-like growths (Hanssen *et al*, 1995). The risk of breast cancer in patients suffering with Cowden syndrome is between 30-50% by the age of 50 (Radford *et al*, 1996). The PTEN protein is an important regulator of the PI3K/Akt pathway and has also been shown to regulate p53 activity (Wan *et al*, 2003; Freeman *et al*, 2003). To date, mutations of the *PTEN* gene have not been linked to increased breast cancer susceptibility outside families affected by Cowden Syndrome (Sakorafas *et al*, 2000).

1.2 Oestrogen Receptor & Breast Cancer

1.2.1 ER Structure & Function

Oestrogen function is mediated by the oestrogen receptor (ER), a member of the steroid/thyroid/retinoid nuclear receptor superfamily of transcription factors. Oestrogen binding to ER induces conformational changes in the receptor which in turn leads to transcriptional activation of oestrogen-regulated genes. The ligand-bound receptor

dimerizes before binding to small palindromic DNA sequences (oestrogen response elements (EREs)) at the promoters of such genes. This triggers the recruitment and interaction with a number of co-activators leading to the assembly of the transcriptional machinery (Nilsson *et al*, 2001). Genes containing such oestrogen response elements are involved in an array of cellular processes which promote cellular growth. These may include gene important in regulating cell cycle progression such as Cyclin A or by promoting translation and survival via the PI3K/Akt pathway (Zilli *et al*, 2009).

There are two oestrogen receptors: ERα and ERβ. ERβ was not identified until 1996 and its exact roles are still to be fully understood. The two receptors share a high degree of homology in certain domains, are both activated by oestrogen and inhibited by tamoxifen (Figure 1.2). ERα and ERβ are produced by distinct genes located on chromosomes 6 and 14, respectively. The differences between the two receptors suggest they may have separate functions particularly in terms of gene transcription and cellular mechanisms activated (Zilli *et al*, 2009).

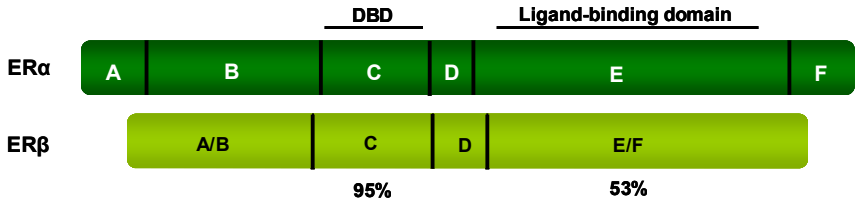


Figure 1.2 Oestrogen structural domains. The DNA-binding and ligand-binding domains are highlighted. The percentage values represent the similarities of each domain in the two receptors.

The oestrogen receptors are composed of three functional domains: the NH_2-terminal domain (A/B); the DNA-binding domain (C) and the ligand-binding domain (D/E/F) (Figure 1.2). The ligand-binding domain (LBD) at the COOH-terminus contains the activation function 2 (AF2) region whose activity is directly regulated by oestrogen binding (Herynk *et al*, 2004). Besides mediating ligand binding, the LBD also plays a part in receptor dimerization and nuclear translocation. The N-terminal domain is involved in protein-protein interactions and harbours the ligand-independent activation function 1 (AF1) region (Nilsson *et al*, 2001). AF1 activity is mediated by phosphorylation and does not require the presence of ligands. The DNA-binding domain (DBD) also has an important role in receptor dimerization and binding to specific DNA sequences. This region contains a two zinc finger structure which mediates binding to the EREs sequences at the gene

promoters (Ali *et al*, 2002). The DBD and LBD of ERα and ERβ are highly homologous suggesting the two receptors are equally efficient at recognising and binding to EREs therefore promoting transcription. On the other hand, the AF1 domain appears to be active only in ERα which may account for the different ligand responses of the two receptors. The C and E domains contain a divided dimerisation domain. The two oestrogen receptors are able to form homo- and heterodimers and these two structural regions are thought to be involved in this process (Herynk *et al*, 2004).

(i) Role of Transcription Factors

Transcriptional regulation is crucial in maintaining control of cellular processes such as growth and differentiation. Transcription is mainly carried out by RNA polymerase but a number of other factors essential for successful transcriptional activation (Lee *et al*, 200). These transcription factors are involved in recognising promoter sequences, mediating conformational changes and assembling the transcriptional machinery. They are characterised by the presence of two main domains, a DNA-binding domain which recognises and binds to specific DNA sequences and a transactivation domain responsible for recruiting and binding other proteins necessary for transcription (Lee *et al*, 2000; Hahn *et al*, 2004). Figure 1.3 depicts the recruitment of cofactors and coregulators at the start of transcription.

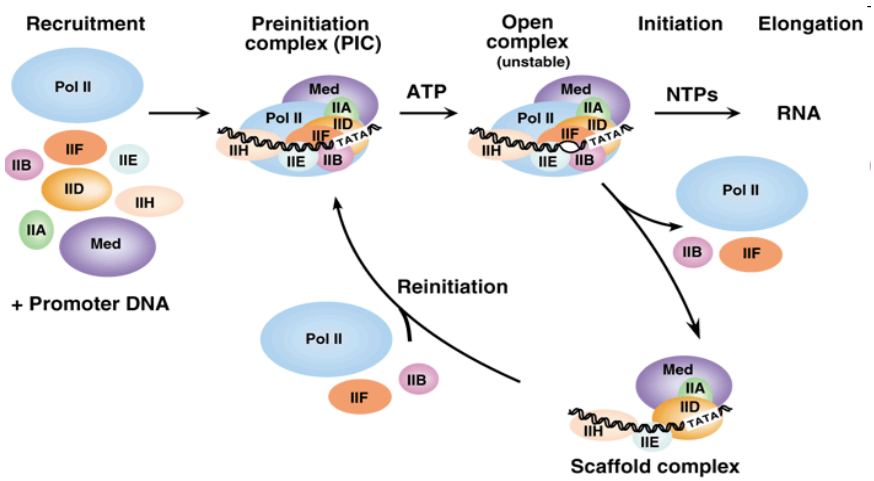


Figure 1.3 Transcription initiation. RNA polymerase II requires a number of different transcription factors and co-activators. TFIIA blocks transcription inhibitors and stabilises binding to DNA; TFIIB binds Pol II and promoter DNA to help fix transcription start site; TFIID is a platform for assembly of TFIIB and TFIIA; TFIIE stabilises transcription complex; TFIIF is responsible for recruiting Pol II to the pre-initiation complex; Mediator (Med) and TFIIH are important to the their kinase, and respective acetyltransferases and helicase activities (Hahn *et al*, 2004).

1.2.2 Oestrogen Mediated Transcription

The AF2 domain interaction surface is composed of six important aminoacids (helix 3, 4, 5 & 12) as shown by crystallographic studies. Oestrogen binding alters the position of helix 12 so it forms the surface for co-activator interaction. ER recruits the general transcriptional machinery thereby promoting gene transcription (Brzozowski *et al*, 1997). Co-activator complexes mediate the interaction between ER and the general transcriptional machinery and enable the recruitment of the ATP-dependent chromatin remodelling complexes such as SWI/SNF (switch/sucrose non-fermenting), the TRAP/DRIP/SMCC, the CREB-binding protein and p300/CBP associated factor (PCAF). Recruitment of histone acetyltransferases and methyltransferases present in these complexes lead to hyperacetylation of histones which is associated with actively transcribed regions. Therefore, they are crucial in facilitating access of the RNA polymerase II and the remaining members of the transcriptional machinery (Ali *et al*, 2002).

The ER is also able to repress gene expression in response to antagonists. In the presence of such compounds (e.g. tamoxifen), the co-activator interaction surface is occluded and co-repressors become associated with ER. Nuclear receptor co-repressor (NCOR) and silencing mediator of retinoid and thyroid receptor (SMRT) recruit histone deacetylases which repress gene transcription (Nilsson *et al*, 2001).

The well characterized p160 co-activator family consists of three members: SRC-1 (p160-1, N-CoA1), TIF2 (SRC-2, GRIP1, N-CoA2) and AIB1 (SRC-3, P/CIP, ACTR, RAC3, TRAM1) (Nilsson *et al*, 2001) (Figure 1.4). All three members interact with a number of different nuclear receptors such as ER, the progesterone receptor (PR), and the thyroid receptor (TR). They bind to the receptors in a ligand dependent manner and increase the receptors ability to activate gene transcription (McKenna *et al*, 1999). Both SRC-1 and AIB1 contain histone acetyltransferases (HAT) activities and all three members of the SRC family directly or indirectly recruit a number of co-activators and other proteins that play an important role in chromatin remodelling, the assembly of the transcription machinery and transcription initiation (Xu *et al*, 2003) (Figure 1.4). The p160 co-activators are widely expressed in different tissues such as brain, liver and testis and share an overall 50% sequence similarity. All three co-activators contain three conserved LXXLL motifs responsible for mediating interaction with ER. They also contain two distinct activation domains involved in recruiting CBP/p300 co-activators, acetyltransferases and co-activator-

associated arginine methyltransferase (CARM1). *In vivo* studies using knockout have shed some light on the individual contributions of each member of the p160 co-activator family. Mice lacking functional SRC-1 protein exhibited normal growth and fertility but oestrogen driven uterine growth appeared to be compromised. Furthermore, SRC-1 depletion reduced transcriptional activity of a number of steroid receptors (Xu *et al*, 1998). TIF2 knockout mice are also viable however they do display reproductive abnormalities (Mukherjee *et al*, 2007). Unlike SRC-$1^{-/-}$ and $TIF2^{-/-}$, AIB1 knockout mice displayed growth retardation, reduced mammary gland alveolar formation and ovulation (Xu *et al*, 2000). On the other hand, double-knockout mice did not survive after birth suggesting functional redundancy of the p160 co-activators may be an important feature of this family (Nilsson *et al*, 2001; McKenna *et al*, 1999; Xu *et al*, 2003). Nevertheless, several studies have demonstrated these molecules retain a certain level of specificity. For instance, the progesterone receptor is preferentially regulated by SRC-1 whilst the glucocorticoid receptors mainly use TIF2 (Li *et al*, 2003).

Members of the p160 co-activator family have also been implicated in breast cancer. The finding that AIB1 enhances oestrogen and progesterone receptor mediated transcription first pointed towards a potential role in breast carcinogenesis (Xu *et al*, 2003). AIB1 has since been found to be amplified and overexpressed in breast cancer (Anzick *et al*, 1997). Furthermore, AIB1 depletion inhibited epidermal growth factor receptor activation as well as reducing oestrogen driven proliferation (Torres-Arzayus *et al*, 2006). SRC-1 expression is also increased in breast cancer despite the minimal expression of this co-activator in human mammary cells (Qin *et al*, 2008). Additionally, SRC-1 expression is associated with HER2 positivity, disease recurrence, metastasis and resistance to endocrine therapies (Redmond *et al*, 2009; Wang *et al*, 2009; Fleming *et al*, 2004). The role of TIF2 in breast carcinogenesis is not as well defined. Recent studies suggest TIF2 RNAi significantly inhibited growth of the MCF-7 breast cancer cells, decreased cell cycle progression and promoted apoptosis suggesting this co-activator may be important in breast carcinogenesis (Karmakar *et al*, 2009).

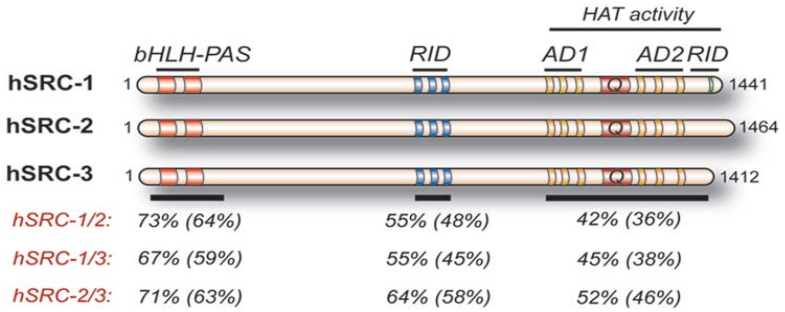


Figure 1.4 Structures of the p160 co-activator family members (Reproduced from Xu *et al*, 2003)

1.2.3 ER Genomic & Non-Genomic Effects

ERα is phosphorylated in response to oestrogen binding and specific signalling pathways. Phosphorylation of ERβ appears to be activated by the MAPK pathway but the effects of estradiol have not been fully determined (Nilsson *et al*, 2001). The AF1 domain at the N-terminus is activated in the absence of ligand as first described by Lee *et al* (1989). It has been proposed that this domain is silenced due to the steric hindrance of the ligand binding domain which is reversed in the presence of oestrogen. Conversely, AF1 activation relieves AF2 repression. The two domains are thought to be able to activate transcription independently or act synergistically. However, whilst the AF1 domain plays a role in both ligand-dependent and independent ERα activation, AF2 is only able to stimulate transcription in response to ligand (Lannigan *et al*, 2003).

The ligand dependent pathway requires direct interaction of oestrogen with the AF2 domain. This activates the receptor leading to the phosphorylation of specific residues (Serine 106, 104 and 118) (Figure 1.5). These residues are highly conserved and once phosphorylated induce ERα mediated gene transcription (Lannigan *et al*, 2003). However, Joel *et al* (1995) have shown that Ser118 appears to be the major site of phosphorylation following ligand activation.

ERα is also phosphorylated in the absence of ligand activation (Figure 1.5). This process is mediated via phosphorylation of residues in the AF1 domain by a number of signalling pathways such as MAPK and Akt (Figure 1.5). This mechanism bypasses the need for oestrogen therefore it has been suggested that it may play a part in endocrine resistance.

Proposed models suggest a number of ways in which ERα may confer resistance: increased ERα activation in the absence of oestrogen, hypersensitivity to low levels of circulating or antagonist driven activation rather than the expected inhibition (Nilsson *et al*, 2001).

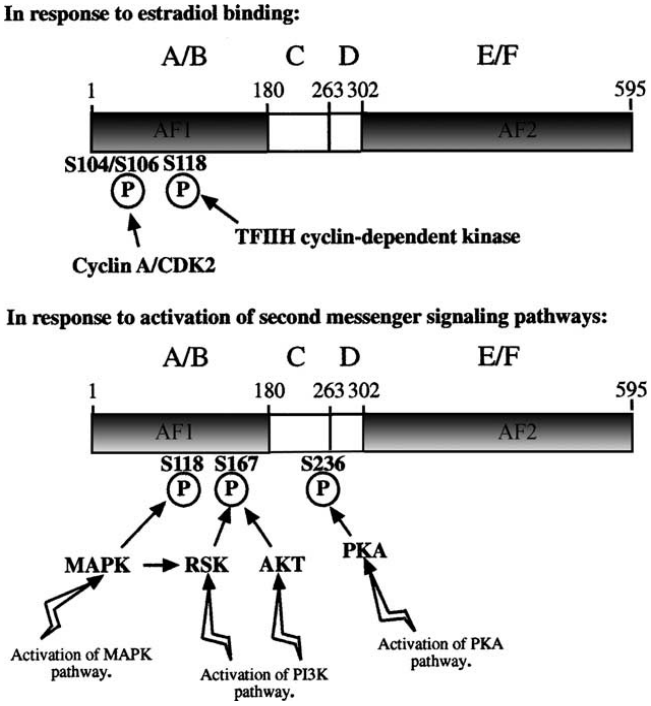


Figure 1.5 Phosphorylation of ERα. This oestrogen receptor may be phosphorylated in the presence of oestrogen or by protein kinases (Reproduced from Lannigan *et al*, 2003)

Besides mediating gene transcription, oestrogen is also able to rapidly activate a number of signal transduction pathways (non-genomic pathway) (Figure 1.6). This is thought to be mediated by a small fraction of ERα or ERα transcriptional variants found near the plasma membrane (Arpino *et al*, 2008). Membrane ERα binds to membrane proteins of lipid rafts thus becoming attached to the inner face of the cellular plasma membrane (Razandi *et al*, 2002). Studies suggest membrane ERα forms a complex including receptor tyrosine kinases (EGFR, HER2 and IGFR) and G-coupled proteins leading to activation of downstream signalling pathways (Razandi *et al*, 2004). Elevated MAPK and PI3K/Akt phosphorylation is able to activate ERα and recruit co-activators promoting gene transcription. The precise mechanisms regulating this process remain unclear (Arpino *et al*, 2008).

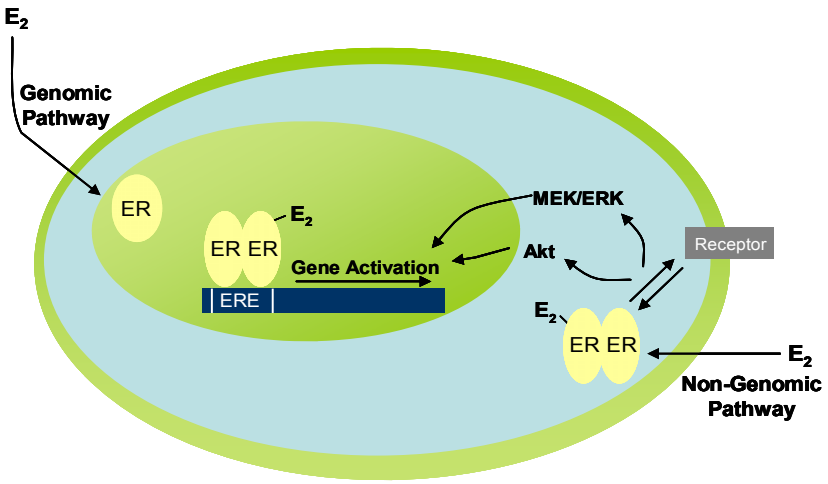


Figure 1.6 Oestrogen-regulated gene transcription. The oestrogen receptor is able to directly bind to DNA sequences and thereby activate gene transcription (genomic pathway). On the other hand, ERα is also able to activate protein kinases such as Akt and MEK/ERK which phosphorylate the receptor and induce transcription.

1.2.4 Ligand Independent Activation: Role of Signalling Pathways

(i) Epidermal Growth Factor (EGF) Receptor Family

The epidermal growth factor family of transmembrane receptor tyrosine kinase is comprised of four members EGFR (HER1), HER2 (erbB2), HER3 (erbB3) and HER4 (erbB4) (Figure 1.7). Ligand binding induces receptor dimerization followed by phosphorylation of tyrosine residues found at the SRC homology 2 (SH2) domain (Yarden *et al*, 2001). These phosphorylated residues trigger the recruitment of adaptor proteins and signal transducers thereby activating a number of signalling pathways regulating cellular processes such as proliferation, motility and apoptosis (Olayioye *et al*, 2000). A variety of ligands have been shown to activate EGF receptors. Despite this, the ligands have non-overlapping functions (Figure 1.7). TGFα and EGF only bind to EGFR whilst neuregulin (heregulin) activates the HER3 and HER4 receptors (Jones *et al*, 1999). Inter-receptor interaction is crucial as HER3 has been shown to have no intrinsic kinase activity whereas HER2 has no identified ligand (Guy *et al*, 1994; Klapper *et al*, 1999). The specific signalling pathways activated and the "strength" of the signal is determined by the identity of the ligand and the receptors involved in the dimeric complexes. Adaptor proteins which associate with the phospho-tyrosine residues are also important in determining the downstream signalling pathways that become engaged (Olayioye *et al*, 1998).

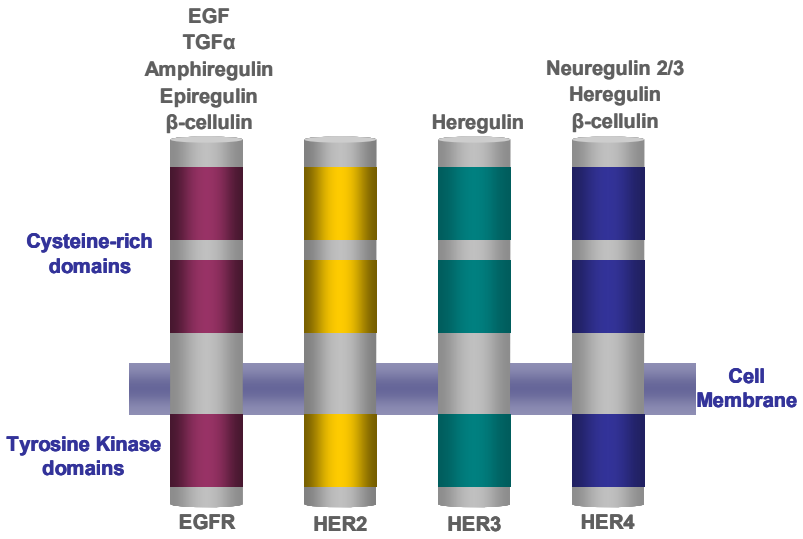


Figure 1.7 EGF receptor family and specific ligands. These comprise of a hydrophobic transmembrane domain, a tyrosine kinase domain found at the cytoplasmic side of the membrane and an extracellular ligand-binding domain.

Epidermal growth factor receptors have often been implicated in cancer including breast cancer (Kurokawa *et al*, 2003). This pathway may become hyperactivated by a number of mechanisms such as receptor/ligand overexpression or constitutive activation. TGFα and heregulin are expressed in breast cancer tissues and have been linked to mammary carcinogenesis (Humphreys *et al*, 2000; Ram *et al*, 2000). Altered receptor expression has also been implicated in breast cancer. Overexpression of EGFR and its ligands are associated with reduced overall survival and anti-oestrogen resistance in breast cancer (Yarden *et al*, 2001). Mutations rendering EGFR constitutively active, due to the deletion of the extracellular domain, are also often reported in breast cancer (Moscatello *et al*, 1995).

HER2 is probably the best studied member of the EGF receptor family. HER2 is overexpressed in 15-30% of invasive ductal carcinomas (Ellis *et al*, 2004). Elevated HER2 expression has been linked to reduced survival and resistance to endocrine therapies and chemotherapy. Moreover, HER2 overexpression is also associated with tumour characteristics such as size, grade and spread to lymph nodes (Ross *et al*, 1998). Despite being devoid of intrinsic kinase activity, HER3 overexpression is found in 17-52% of breast carcinomas (Hamburger, 2008). This does not appear to be a result of gene amplification or mutation (Lemoine *et al*, 1992). High HER3 expression is positively associated with disease recurrence, tumour size/grade and metastasis (Naidu *et al*, 1998; Travis *et al*, 1996). Unlike

the other EGF receptors, HER4 overexpression is associated with increased survival (Frogne *et al*, 2009). Lower HER4 expression is often observed in breast cancer and is indicative of a differentiated phenotype (Kew *et al*, 2000).

(ii) MEK/ERK Pathway

Epidermal growth factor receptors mediate cellular proliferation through a number of downstream signalling molecules such as the MEK/ERK pathway (Kurokawa *et al*, 2000). This pathway regulates several different mechanisms including survival, differentiation and motility (Roberts *et al*, 2007).

Aberrant EGFR and HER2 receptor signalling has been shown to hyperactivate the MEK/ERK pathway (Kurokawa *et al*, 2003). Furthermore, elevated MEK/ERK activation is also demonstrated in long-term oestrogen deprived cell line models (Santen *et al*, 2005; Normanno *et al*, 2006). Studies have shown that activation of this signalling pathway is associated with oestrogen-unresponsive breast cancer cells (Shim *et al*, 2000) and with anti-oestrogen resistance in MCF-7 cells (McClelland *et al*, 2001). MEK/ERK hyperactivity promotes co-activator association with ERα therefore inducing gene expression (Lavinsky *et al*, 1998). Moreover, activation of the MEK/ERK has also been shown to phosphorylate Ser118 in ERα (Kato *et al*, 1995). This induces ligand independent receptor activation thus potentially promoting resistance to anti-oestrogenic therapies.

(iii) PI3K/Akt Pathway

Activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway (Figure 1.8) is often implicated in human breast cancer (Perez-Tenorio *et al*, 2002). Akt signalling pathway regulates a number of cellular processes such as survival, proliferation and migration. Receptor-mediated activation of PI3K leads to the conversion of phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 then proceeds to recruit proteins to the plasma membrane (Figure 1.8) where they become activated (Shtilbans *et al*, 2008). Akt is one of these proteins. Activating mutations in the PI3K gene have been reported in breast carcinomas (Levine *et al*, 2005).

PI3K-mediated Akt activation is further controlled by PTEN (phosphatase and tensin homologue) which removes the 3' phosphate of PIP_3 thus inhibiting phosphorylation of downstream signalling pathways (Dillon *et al*, 2007) (Figure 1.8). Loss of PTEN protein is observed in 30% of breast carcinomas suggesting this protein is important in carcinogenesis

(Garcia *et al*, 2004). Activating mutations and/or PTEN protein loss may be responsible for elevated Akt activation which is associated with tamoxifen resistance and reduced survival.

The Akt protein kinases, also known as protein kinases B (PKBs), consists of 3 isoforms Akt 1 (PKB α), Akt 2 (PKB β) and Akt 3 (PKB γ). The Akt family is evolutionary conserved and the three isoforms share similar structural domains comprising the pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain (Scheid *et al*, 2003). Phosphorylation of threonine 308 (activation loop of the kinase domain) and the serine 473 (hydrophobic motif at the carboxyl domain) residues is required for the full activation of Akt (Hanada *et al*, 2004).

Increased activity and dysregulation of Akt is a common feature in a number of cancers including breast cancers. Although many cancers exhibit a marked increase in constitutively activated Akt, mutations are relatively rare. Studies in human breast cancer patients have shown that Akt activation is often associated with triple negative (ER-, PR- and HER2-negative) breast cancers (Umemura *et al*, 2007) and has been found to be a negative predictor of response to hormone therapy in metastatic cases (Tokunaga *et al*, 2006). Tamoxifen resistant MCF-7 cells express increased phosphorylated Akt levels suggesting this may contribute towards endocrine resistance (Jordan *et al*, 2004). This is further supported by immunohistochemical studies which report a link between Akt activation and reduced survival of breast cancer patients (Generali *et al*, 2008). Elevated Akt signalling is thought to mediate a variety of pathways leading to anti-oestrogen resistance. Shin *et al* (2002) have shown that Akt phosphorylation is associated with cyclin-dependent inhibitor p27 expression. Akt contributes towards cellular proliferation by phosphorylating p27, relieving its inhibitory effect of cyclin A (CDK2) thereby promoting cell cycle entry (Shin *et al*, 2002). Other studies have shown Akt is able to inhibit apoptosis by phosphorylating BAD (Franke *et al*, 1997) whilst it also appears to play a part in regulating cellular motility and invasion by influencing actin organization and enhancing matrix-metalloproteinase 2, a promoter of cellular invasion (Chin *et al*, 2009). Importantly, Akt is associated with epidermal mesenchymal transition (EMT) which promotes tumour progression to invasive and metastatic carcinomas (Grille *et al*, 2003).

Although they share structural domains, Akt isoforms appear to mediate different cellular processes. Whereas Akt 1 is ubiquitously expressed in most tissues, Akt 2 is mainly expressed in insulin target tissues such as the liver (Ju *et al*, 2007; Hanada *et al*, 2004). Akt

3 is not as widely expressed as the other Akt isoforms and is mainly expressed in the brain and testis (Easton *et al*, 2005). Mouse model studies have shown isoform deletion result in viable animals however they do show reduced growth/weight and altered glucose regulation after Akt 1 and Akt 2 knockouts, respectively (Cho *et al*, 2001; Cho *et al*, 2001a). The fact that individual isoform deletions give rise to viable animals indicates that there may be some levels of redundancy and individual isoforms may compensate for the absence of any of the other members. Alternative kinases able to substitute for Akt function have also been identified and are thought to mediate the same pathways regulated by the Akt pathways (Brunet *et al*, 2001; Liu *et al*, 2001; Franke *et al*, 2003)

All three Akt isoforms have been implicated in breast cancer despite regulating distinct cellular processes. Akt 1 induces the secretion of metalloproteinases thus promoting tumour invasion and metastasis (Larue *et al*, 2005; Thant *et al*, 2000) whilst Akt 2 appears to play an important role in the insulin pathway and is mainly expressed in insulin target tissues such as the liver (Hanada *et al*, 2004). Inactivating somatic *Akt 1* mutations are present in 8% of breast carcinomas (Carpten *et al*, 2007) and Akt 1 siRNA experiments have been shown to enhance breast cancer cell line migration and invasion (Irie *et al*, 2005). On the other hand, reports suggest that Akt 2 overexpression is linked to increased levels of β1-integrin leading to higher invading ability of human breast cancer cells (Arboleda *et al*, 2003). Activating Akt 2 kinase domain mutations and Akt 2 gene amplification have been identified in breast cancer (Bellacosa *et al*, 1995 & 2005) and Akt 2 downregulation is associated with increased apoptosis (MacKeigan *et al*, 2005). These results indicate Akt 1 and Akt 2 may have specific, perhaps even opposing, non-redundant roles in breast cancer. The role of Akt 3 is not as clear, however there is evidence that this isoform may be involved in breast tumourigenesis (Dillon *et al*, 2007). Akt 3 expression is often upregulated in more advanced, ER-negative breast carcinomas (Zinda *et al*, 2001; Nakatani *et al*, 1999). Nevertheless, constitutive activation of Akt 3 in MCF-7 cells results in oestrogen independent tumour growth (Faridi *et al*, 2003) suggesting this isoform may be involved in endocrine resistance.

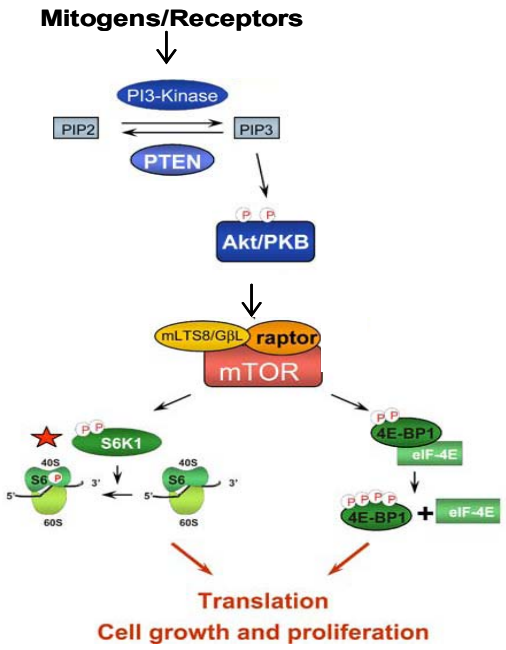


Figure 1.8 PI3K/Akt Pathway. PIP_3 is generated by PI3K and it leads to the phosphorylation of Akt which in turn activates mTOR and ERα. The PI3K/Akt pathway regulates a number of cellular processes such as growth, survival and motility (Reproduced from Hynes *et al*, 2006)

Downstream components are also important in the PI3K/Akt pathway. The mammalian target of rapamycin (mTOR) is activated by Akt (Figure 1.8) and is associated with tamoxifen resistance in breast cancer (Hidalgo *et al*, 2000). mTOR is a serine/threonine kinase that regulates the function of p70S6 kinase and 4E-BP1 (Figure 1.8) which are involved in the control of transcriptional activation. Furthermore, Akt is also able to phosphorylate ERα in the absence of oestrogenic stimuli (Lannigan *et al*, 2003). Therefore, increased Akt activation is able to promote ligand-independent ERα phosphorylation and ERα-driven gene transcription.

(iv) IGFR Signalling

The insulin-like growth factor (IGF) system has also been linked to growth and survival of breast cancer cells (Jerome *et al*, 2003). Activation of this receptor stimulates cellular proliferation and survival. The IGF I receptor (IGFR) possesses ligand-activated tyrosine kinase activity hence ligand binding induces conformational changes in the intracellular domain followed by auto-phosphorylation (Sepp-Lorenzino *et al*, 1998). This then triggers the phosphorylation of the insulin receptor substrate 1 (IRS-1) which acts as a docking site

for numerous SH2 domain containing proteins (Zhang *et al*, 2000). PI3K is amongst the proteins binding to IRS-1 and thus becoming activated. This suggests that the downstream Akt protein is also regulated by IGF signalling (Jerome *et al*, 2003). There are two ligands in the IGF signalling system, IGFI and IGFII, the former of which acts as a mitogen in breast cancer cells including MCF-7 cells (Ullrich *et al*, 1986). IGF signalling is quite complex particularly since IGFR and the insulin receptor are able to dimerize giving rise to a number of hybrid receptors (Leroith *et al*, 2003). Prospective studies have observed a correlation between circulating IGF I levels and the risk of developing breast cancer but only in pre-menopausal women (Hankinson *et al*, 1998)

1.3 Current Therapies in Breast Cancer

1.3.1 Surgery

For the last decade, surgery has been at the forefront of breast cancer treatment. The procedures, however, have evolved due to technological advances and new therapeutic approaches. The adequate type of surgery is based on the stage and type of the breast cancer and its aggressiveness (Dixon *et al*, 2002). Breast-conserving surgery (or lumpectomy) involves the removal of the tumour and some of the surrounding tissue. Larger tumours, on the other hand, require the removal of all breast tissue (mastectomy) (Dixon *et al*, 2002). Patients are often treated with hormone therapies and/or chemotherapy after surgery (adjuvant) if there is evidence the cancer may have spread thereby reducing the changes of recurrence. Furthermore, some patients also receive such therapies before any surgical procedure (neo-adjuvant) (Houssami *et al*, 2006). This approach can allow either surgery in otherwise inoperable tumours or breast-conserving surgery instead of a mastectomy.

1.3.2 Chemotherapy & Radiotherapy

Radiotherapy is an important component of breast cancer treatment that relies on the use of radiation to damage cancer cells. All cells are susceptible to radiation however cells dividing at a fast rate, such as cancer cells, are more sensitive to it. Radiotherapy is routinely used following surgery allowing patients to retain their breast whilst also reducing the chances of recurrence (Early Breast Cancer Trialists' Collaborative Group, 2005). Radiation is routinely applied externally but a less common method of internal radiation (brachytherapy) is also used with radioactive "seeds" which are temporarily placed at the tumour site (Patt *et al*, 2005).

Similarly to radiotherapy, chemotherapeutic drugs target rapidly diving cells but unlike radiation, chemotherapy is a systemic drug treatment. These drugs may be used in an adjuvant and neoadjuvant setting as they reduce tumour size prior to surgery and minimise the risk of recurrence. Chemotherapy is also used in recurrent breast cancer. There are different classes of drugs available targeting distinct cellular processes. Alkylators, such as cyclophosphamide, reduce cell growth; antimetabolites (e.g. 5' fluorouracil) interfere with DNA production; antimitotic and antimicrotubule agents hinder the cells ability to undergo mitosis and affect cellular structure, respectively. Finally, there are also antibiotic drugs, such as doxorubicin and mitomycin C, that inhibit gene replication. These agents are often used in combination depending on the patient's needs.

1.3.3 Selective Oestrogen Receptor Modulators

Once the role of oestrogen in breast carcinogenesis was established new therapies started being developed to target ER which mediates oestrogen function. The most widely used SERM is Tamoxifen which was first used in the 1970s and remains a crucial part of endocrine treatment for breast cancer patients (Figure 1.9). Tamoxifen acts as an antagonist as it competes with oestrogen for binding to the AF-2 domain of the receptor. This mechanism is further mediated by the ability of tamoxifen to repress co-activator binding (Shiau *et al*, 1998) and recruit co-repressors to the complex (Lavinsky *et al*, 1998). Oestrogen receptor activity in the breast epithelial is mainly mediated by the AF2 region hence tamoxifen acts as an antagonist. However, in other tissues where the AF1 activity is more significant, tamoxifen becomes an agonist (Metzger *et al*, 1995) (Figure 1.9).

The roles of tamoxifen have increased throughout the years as a number of studies have shown it may play a role in prevention and be useful in a neo-adjuvant setting. It is routinely used in both pre and post menopausal women whose tumours are ER-positive. The effects of tamoxifen on ER-negative tumours appear to be limited. Tamoxifen has been shown to be particularly effective in preventing tumour recurrence as described by the Early Breast Cancer Trialists' Collaborative Group. This study conclusively showed that annual recurrence decreased by 47% following 5 year treatment with tamoxifen. In addition, they also observed an overall reduction in mortality (26%) (Early Breast Cancer Trialists' Collaborative Group, 1998). Metastatic breast cancers may also be treated with tamoxifen as 20% of women experience a 6 month reduction in disease progression (Osborne *et al*,

1998). Tamoxifen is also useful in a preventive setting. Studies such as the National Surgical Adjuvant Breast and Bowel Project revealed that tamoxifen reduced the risk of invasive and non-invasive cancer by 50%. They also observed a 69% overall decrease in the occurrence of ER-positive tumours but it did not affect the occurrence of ER-negative tumours (Fisher *et al*, 1998).

Some adverse effects have been reported following tamoxifen treatment such as an increased incidence of endometrial cancer and benign uterine disease (Nordenskjöld *et al*, 2005). Further studies have also observed an increase risk of stroke, deep-vein thrombosis and pulmonary embolism in tamoxifen treated patients (Fisher *et al*, 1998). On the other hand, the Scottish Cancer Trials Breast Group has shown that tamoxifen reduced the risk of myocardial infarction (McDonald *et al*, 1995). This was further confirmed by more recent studies which observed a 30% reduction in the risk of coronary disease after 5 year treatment with tamoxifen in comparison to 2 year treatment (Nordenskjöld *et al*, 2005).

Despite the success of tamoxifen its agonistic effects and the development of resistance are areas of concern. As a result, second and third generation SERMs have been developed. Raloxifene was first approved for the treatment of osteoporosis but is also an oestrogen antagonist (Figure 1.9). Unlike tamoxifen, the anti-oestrogenic effects of raloxifene are observed in breast epithelial and in the endometrium hence there is no reported increase in the risk of endometrial cancer (Gasco *et al*, 2005; Ariazi *et al*, 2006). These results suggest that raloxifene may be a safer alternative to tamoxifen in the prevention setting. The MORE trial showed that raloxifene reduced the risk of in situ and invasive breast cancer by 60% in comparison to a placebo control (Dickler *et al*, 2001). Raloxifene was also found to be as effective as tamoxifen according to the STAR trial which compared the two drugs in 20,000 women (Howell, 2008). Toremifene is another SERM routinely used in treating breast cancer, though this particular drug is often used in metastatic cases (Holli *et al*, 2000).

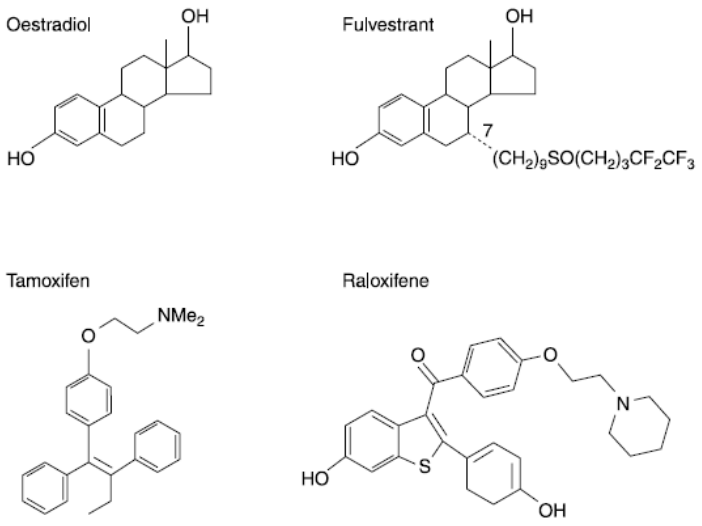


Figure 1.9 Chemical structures of oestradiol and SERMs tamoxifen, raloxifene and fulvestrant (Reproduced from Howell, 2006).

Unlike the above SERMs, faslodex (fulvestrant; ICI 180,780) is considered a pure anti-oestrogen (Figure 1.9) as it prevents the activation of both AF1 and AF2 domains therefore acting as a true antagonist (Ali *et al*, 2002). Faslodex has also been shown to inhibit receptor dimerisation, reduces ER shuttling to the nucleus and increases protein turnover (Osborne *et al*, 1995; Dauvois *et al*, 1993; Dauvois *et al*, 1992). Like other SERMs, faslodex competitively binds to the oestrogen receptor but its affinity is much stronger than that of tamoxifen (Wakeling *et al*, 1991). Faslodex is recommended for the treatment of ER-positive metastatic breast cancer, particularly in patients that have developed anti-oestrogen resistance (Buzdar *et al*, 2001). Two randomised trials have shown that faslodex is as effective as the aromatase inhibitor anastrozole in reducing tumour progression and in promoting overall survival. In addition, tamoxifen-resistant patients responded to faslodex treatment suggesting a lack of cross-resistance (Howell *et al*, 2002; Osborne *et al*, 2002). Since faslodex does not promote agonistic effects it has not been associated with an increase in endometrial cancer (Addo *et al*, 2002). These results suggest faslodex may be a good alternative to other anti-oestrogenic therapies.

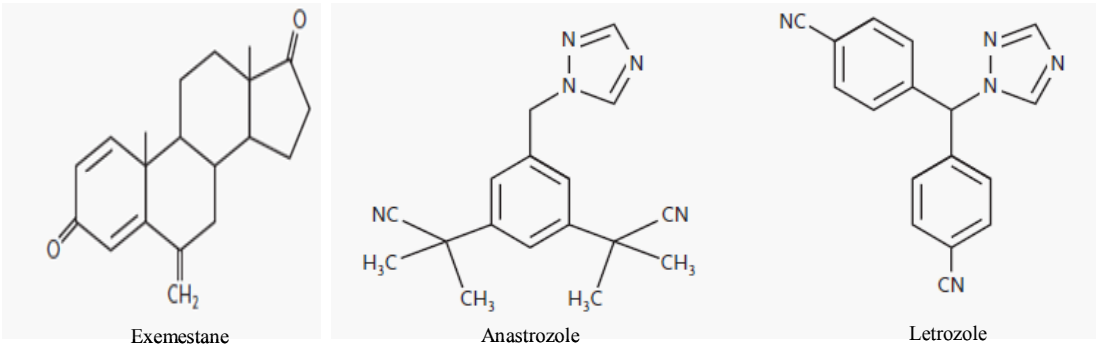
Despite the positive results, resistance to faslodex has been reported in cell line models (Shaw *et al*, 2006). Increased HER signalling has been implicated in this process and it appears that direct ER modulation may not be important (McClelland *et al*, 2001). These results suggest that resistance to faslodex in a clinical setting is more than likely.

1.3.4 Aromatase Inhibitors

Aromatase inhibitors are an alternative to tamoxifen in treating post-menopausal ER-positive breast cancers and as preventive approach. Such drugs reduce oestrogen levels by inhibiting or inactivating aromatase, a member of the cytochrome P-450 enzyme superfamily responsible for synthesising oestrogen from androgenic substrates (Smith *et al*, 2003). The first aromatase inhibitors were proven to not be specific or powerful enough. The three currently available aromatase inhibitors (Figure 1.10) (anastrozole, letrozole and exemestane) are third-generation aromatase inhibitors (Macedo *et al*, 2009). There are two types of aromatase inhibitors, type I inhibitors are regarded as enzyme inactivators since they irreversibly bind to the aromatase molecule therefore blocking androstenedione from binding. Exemestane falls into this category. Type II inhibitors (anastrozole and letrozole), on the other hand, reversibly bind to aromatase (Smith *et al*, 2003) (Figure 1.10).

Clinical trials testing anastrozole against tamoxifen have found that treatment with the aromatase inhibitor was associated with a decrease in tumour progression as well as a reduction in certain side effects such as thromboembolic events and vaginal bleeding (Nabholtz *et al*, 2000). The ATAC trial also reported a significant increase in disease-free survival in patients treated with anastrozole in comparison to tamoxifen alone whilst other trials have suggested that anastrozole may be effective in a neo-adjuvant setting (Smith *et al*, 2005).

A study conducted by the International Letrozole Breast Group has shown that similarly to anastrozole, letrozole treated patients had higher survival rates than those treated with tamoxifen alone (Mouridsen *et al*, 2001). Studies on exemestane, on the other hand, suggest this drug may be useful in metastatic breast cancer patients (Paridaens *et al*, 2000).



Exemestane Anastrozole Letrozole

Figure 1.10 Chemical structures of aromatase inhibitors (Adapted and reproduced from Smith *et al*, 2003)

1.3.5 Monoclonal Antibodies

Members of the epidermal growth factor receptor (EGF) family such as EGFR and HER2 have been implicated in breast cancer development and have therefore become therapeutic targets. Monoclonal antibodies generated to specifically bind to these receptors have been generated and are able to block its function consequently inhibiting cell growth and survival.

(i) Anti-HER2

The *HER2* gene is amplified or overexpressed in approximately 30% of all breast carcinomas and in some other types of cancers (Yu *et al*, 2000). This gene encodes the 185 kDa transmembrane tyrosine kinase receptor HER2, a member of the EGF family. Ligand binding leads to dimerization and consequent activation of such receptors which in turn activate a wide network of signalling pathways (Hudis, 2007). *HER2* overexpression significantly correlates with reduced survival in breast cancer patients whilst it also appears to be a predictive marker for response to breast cancer treatments (Bange *et al*, 2001). Several studies have reported that *HER2* overexpression was associated with lack of response to endocrine treatments (Sjogren *et al*, 1998; Borg *et al*, 1994) as well as chemotherapeutic approaches (Jarvinen *et al*, 1998; Gusterson *et al*, 1992).

Herceptin (trastuzumab) was the first licensed monoclonal antibody used for targeted therapies. It comprises of a human IgG antibody with two antigen-specific sites that recognise and competitively bind to the extracellular domain of the HER2 receptor (Hudis *et al*, 2007). The exact mechanisms of herceptin function has not been fully determined but it has been proposed that this antibody may potentially interfere with receptor dimerization, induce an immune response leading to cell death or downregulate receptor levels due to increased endocytosis. Further studies have also suggested herceptin inhibits the MAPK and PI3K/Akt pathways (Jackson *et al*, 2004; Mohsin *et al*, 2005).

Herceptin was first approved in 1998 and has proven to be effective in treating HER2-positive metastatic breast cancer either alone (Vogel *et al*, 2002; Baselga *et al*, 2005) or in combination with chemotherapeutic agents (Slamon *et al*, 2001; Marty *et al*, 2005). The National Surgical Adjuvant Breast and Bowel Project and the North Central Cancer Treatment group trials compared adjuvant chemotherapy with or without concurrent herceptin following surgical removal of HER2-positive breast cancer. These studies reported a reduction in the risk of death (33%) and improved outcome in patients treated

with herceptin in combination with paclitaxel after chemotherapy (Romond *et al*, 2005). Treatment with adjuvant herceptin alone after adjuvant chemotherapy has also been linked to an increase in disease-free survival after a year of treatment (Piccart-Gebhart *et al*, 2005). Despite remaining HER2-positive, some tumours do not respond to herceptin treatment hence new combination treatments were explored. Herceptin in combination with tamoxifen reduced growth in BT 474 cell line by 44% (higher than each individual treatment) (Ropero *et al*, 2004). Furthermore, this combination appears to inhibit HER2 activity and induce G0-G1 cell cycle accumulation (Wang *et al*, 2005).

2C4 (Pertuzumab) is another available monoclonal antibody targeting the HER2 receptor. This antibody sterically blocks HER2 homo- and heterodimerization with EGFR and HER3 thereby inhibiting the activation of downstream signalling pathways leading to cellular growth and survival. Similar to herceptin, 2C4 binds to the extracellular domain of HER2 but it has been shown that the two antibodies bind to distinct epitopes (Takai *et al*, 2005). Xenograft studies revealed that 2C4 was able reduce tumour growth as efficiently as herceptin but unlike herceptin this is not dependent on HER2 expression levels since both low and high-HER2 expressing tumours are equally responsive (Agus *et al*, 2002). Clinical studies indicate a variety of tumours may respond to 2C4 treatment and that treatment with this antibody is well tolerated (Agus *et al*, 2005). A number of clinical trials are currently underway in a variety of cancers including ovarian, lung and prostate.

1.3.6 EGF receptor & Tyrosine Kinase Inhibitors

The EGF receptor (EGFR) has been shown to contribute to pathogenesis and tumour progression in breast cancer. EGFR expression is found in 50 to 70% of breast, lung and colon carcinomas. It has been proposed that EGFR expression is a modest prognostic indicator in breast cancer whilst others have shown an inverse correlation between the receptor expression and hormone receptor status (Klijn *et al*, 1992).

In order to target this membrane receptor, small molecule tyrosine kinase inhibitors were developed. These compounds competitively bind the ATP-binding site of the EGFR thereby blocking ATP access (Nahta *et al*, 2004). Furthermore, these agents bind to the intracellular domain of the receptor suggesting they may be effective in inhibiting a truncated form of EGFR (EGFR vIII). This receptor is inaccessible to monoclonal antibodies as it lacks its

extracellular domain. The truncated EGFR is often found in breast cancer and is associated with highly aggressive tumours (Nahta *et al*, 2004).

Gefitinib (Iressa, ZD1839) is a tyrosine kinase inhibitor targeting EGFR. The anti-tumour activity of this highly specific compound is not dependent on the expression levels of the receptor (Moasser *et al*, 2001). It has been proposed that response to anti-EGFR agents may be dependent on the total levels of HER receptors and ligands present in tumours which is further corroborated by the link between response to EGFR inhibitors and HER2/3 expression (Normanno *et al*, 2005). Furthermore, some groups have recently found that herceptin in combination with gefitinib produces a synergistic anti-tumour effect in breast cancer (Normanno *et al*, 2002). Gefitinib has been shown to interfere with a variety of cellular processes. This inhibitor leads to cell cycle arrest (G1), reduced proliferation, elevated apoptosis and it also appears to have anti-angiogenic effect (Ciardiello *et al*, 2002; Hirata *et al*, 2002; Chan *et al*, 2002). Additionally, gefitinib appears to increase growth inhibition when in combination with chemotherapeutic agents and the response to endocrine therapies (Moulder *et al*, 2001).

Tarceva (Erlotinib, OSI-774) is another EGFR tyrosine kinase inhibitor. Similarly to gefitinib, tarceva inhibits tumour progression, promotes cell cycle arrest and apoptosis (Arteaga, 2001; Slichenmyer *et al*, 2001; Elsayed *et al*, 2001). This EGFR inhibitor is well tolerated and induces responses in a variety of cancer including non-small-cell lung cancer, ovarian and breast cancer (Nahta *et al*, 2004). Ongoing clinical trials are investigating the combination of tarceva with HER2-targetting therapies such as herceptin and 2C4 (Mass, 2004). A study from Friess *et al* (2005) reported additive anti-tumour effects of combining tarceva with 2C4 in breast cancer.

Clinical response to EGFR tyrosine kinase inhibitors in metastatic breast cancer has been very limited. This is particularly obvious in patients who have been exposed to a number of treatments such as chemotherapy and endocrine therapies (Nahta *et al*, 2004). These observations suggest there is an intrinsic resistance mechanism in breast cancer tumours. Gefitinib has proven to be a success in treating non-small-cell lung cancer and its efficiency appears to be linked to the presence of specific EGFR mutations (Normanno *et al*, 2005). Such mutations have not been reported in cell lines or primary breast carcinomas.

Additionally, the activation of downstream signalling pathways such as the PI3K/Akt has been observed in the resistance to gefitinib (Ferrer-Soler *et al*, 2007).

1.5 Aims

This project was designed to determine the role of intracellular signalling pathways in conferring resistance to endocrine therapies in breast cancer. In order to achieve this, a three step MCF-7 cell-based model was used. Each cell line differs in their oestrogen-sensitivity and response to anti-oestrogen therapies. Over the years a number of membrane receptors and downstream signalling cascades have been implicated in breast cancer.

It is likely that endocrine resistance results from deregulation of more than a single pathway, particularly when protein interactions and pathway crosstalk are so widely spread. Therefore, this study was divided into two components:

(i) Firstly, the role of epidermal growth factor family was assessed in this cell line model. HER2 transcriptional regulation has been shown to be downregulated following E_2 stimulation in oestrogen-responsive breast cancer cell lines (Newman *et al*, 2000). It has been proposed that this process is mediated by members of the p160 family of co activators, particularly SRC-1. These co activators are responsible for mediating ERα induced transcription but Newman *et al* (2000) suggest that these co activators are also able to directly mediate transcription of certain genes, including *HER2*. Given this evidence, the role of oestrogen-mediated HER2 regulation was established in the MCF-7 variant cells. Furthermore, the effects of oestrogen on the transcription of the other members of the EGF receptor family (EGFR, HER3 & HER4) were also determined. Altered receptor tyrosine kinase transcriptional regulation is likely to contribute towards endocrine resistance.

(ii) The second part of the project focussed on the downstream signalling pathways, particularly the PI3K/Akt pathway. Previous studies have shown that increased Akt activation is often observed in breast cancer and is associated with endocrine resistance (Tokunaga *et al*, 2006). The upstream regulators of Akt activation were also investigated as well as the downstream pathways activated by the Akt kinase. This is of particular interest considering the ability of signalling pathways to activate ERα in the absence of oestrogen (ligand-independent activation) which has been implicated in the advent of endocrine resistance (Lannigan *et al*, 2003).

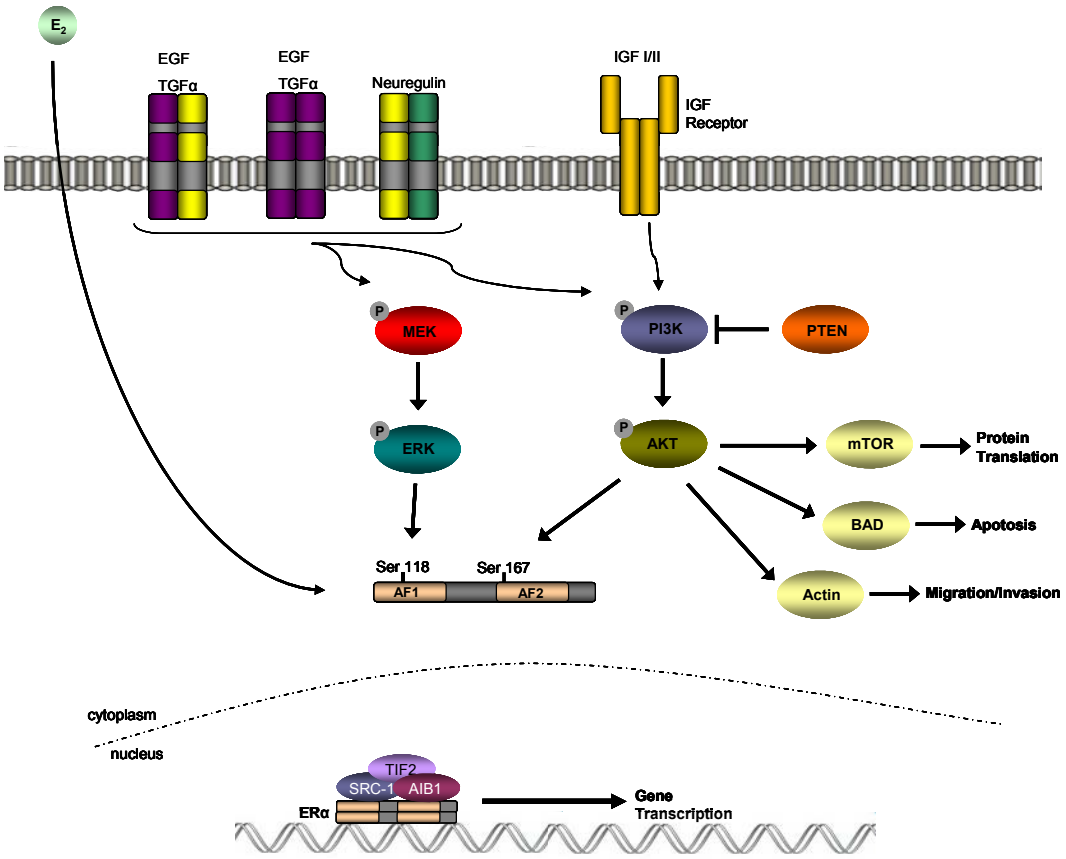


Figure 1.12 Signalling pathways and ERα activation. This diagram depicts the main areas this study will be focussing on and how these aspects interact.

The pathways investigated here regulate a number of cellular processes such as cell cycle entry, migration, invasion and apoptosis. Consequently, these were also studied in order to determine whether these cellular activities are altered in endocrine resistant cell line models.

Primary tumour material was examined in order to test the major findings obtained in the cell line models. This will help evaluate the validity of the *in vitro* studies in comparison to *in vivo*.

Chapter 2

Materials & Methods

2.1 Materials

All chemicals were obtained from Sigma Aldrich unless otherwise stated. Materials are listed according to technique.

2.1.1 Cell Culture

(i) Origin of Cell Lines

In order to study and determine which factors are involved in the development of acquired resistance to anti-oestrogens a range of different breast cancer cell lines were used which were developed and characterized by Professor Robert Clarke's group (Georgetown University, Washington DC).

MCF-7 cells are the most commonly used oestrogen positive breast cancer cell line model. This cell line was first established in 1973, derived from a pleural effusion of a post-menopausal patient with metastatic mammary carcinoma (Soule *et al*, 1973). This cell line is representative of the early stages of the disease and is the ultimate model of oestrogen-dependent and anti-oestrogen sensitive breast cancer.

Oestrogen-independent and tamoxifen resistant cells were derived from the MCF-7 cell line and provide an adequate model mimicking disease progression and endocrine resistance often observed in breast cancer patients (Clarke *et al*, 1994; Han *et al*, 2004). MCF-7 cells were inoculated into ovariectomized mice in order to select for oestrogen independent growth *in vivo* (Clarke *et al*, 1989) (Figure 2.1). The MCF7/LCC1 (LCC1) cell line was isolated from tumours growing in these mice and these cells acquired a hormone independent phenotype. However, this cell line still responds mitogenically to oestrogen stimulation (Clarke *et al*, 1989). LCC1 cells remain anti-oestrogen sensitive and their growth is fully blocked by treatment with such therapies (Brunner *et al*, 1993).

The ICI 182,780 resistant MCF-7/LCC9 (LCC9) cell line was developed following *in vitro* stepwise selection of LCC1 cells (Figure 2.1). These cells were treated with increasingly higher concentrations of ICI 182,780 ranging from 10pM to 1μM (Brunner *et al*, 1997). LCC9 cells are also resistant to other anti-oestrogens such as tamoxifen and are completely oestrogen independent.

Furthermore, ERα negative MDA-MB 231 cells were also used in these studies (Calvo *et al*, 1983). This cell line provides a useful negative control for some of the experiments

performed and helps determine the role of the oestrogen receptor in the resistant cell line models used.

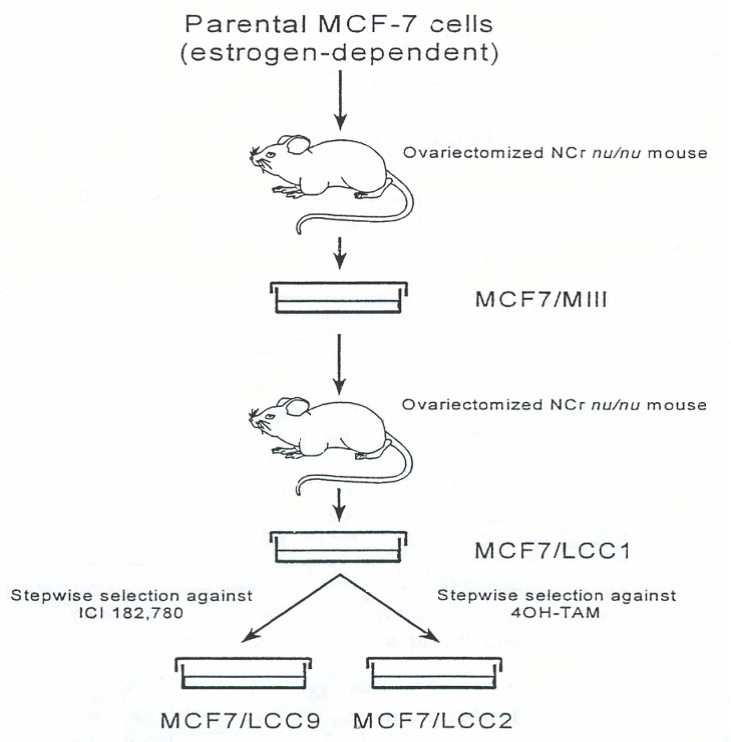


Figure 2.1 Derivation of MCF-7 variant breast cancer cell lines (Reproduced from Brunner *et al*, 1997)

(ii) Cell Lines

The original MCF-7 cell line was obtained from the European Tissue Culture Collection (Porton Down, UK). MCF-7 derived MCF-7/LCC1 and MCF-7/LCC9 cell lines were kindly donated by Professor Robert Clarke (V.T. Lombardi Cancer Research Centre, Georgetown University Medical School, Washington, D.C.). The MDA-MB231 cell line was obtained from the American Type Culture Collections (Manassas, Virginia).

2.2 Solutions

- **Freezing Mix:** 10% DMSO in foetal calf serum.
- **SRB Dye:** 0.4% SRB in 1% Acetic Acid.
- **Lysis Buffer:** 50mM Tris (pH7.5), 5mM EDTA (pH 8.5), 150mM NaCl, 1% Triton X-100, Aprotinin 10µg/ml & 1x protease inhibitor cocktail (Roche).

- 5x Loading Buffer: 125mg Tris Base, 1.25g SDS, 6.25ml β-mercaptoethanol, 12.5 ml glycerol, 417µl bromophenol blue solution made up to total volume of 25ml with dH_2O.

- Tris Buffered Solution: 6.05g Tris base, 8.76 NaCl, made up to 1L with dH_2O adjusted to pH 7.5.

- Vindelovs Solutions

Stock Solution: 200mg Trisodium Citrate, 121mg Tris, 1044mg Spermine Tetrahydrochloride and 2ml Nonidet NP40 in 2L dH_2O; pH adjusted to 7.6.

Solution A: 15mg Trypsin in 500ml Stock Solution

Solution B: 250mg Trypsin Inhibitor and 50mg of RNase A in 500ml of stock solution.

Solution C: 208mg propidium iodide and 500mg Spermine Tetrahydrochloride in 500ml of stock solution.

- Citrate Buffer: 0.1M Citric Acid & 0.7M Sodium Citrate

2.3 Methods

2.3.1 Cell Culture

(i) Routine Cell Culture

The MCF-7 cell line was grown as a monolayer in DMEM (+ phenol red) supplemented with 1% Penicillin/Streptomycin and 10% heat inactivated foetal calf serum (inactivated by incubating at 56ºC for 20 min). MCF-7 cells are grown in the presence of phenol red due to its oestrogenic effects. MCF-7 variant cells were routinely cultured in DMEM (-phenol red) supplemented with 1% Penicillin/Streptomycin, 2mM Glutamine and 5% double charcoal stripped serum (DCSS). All cell lines were maintained in a humidified atmosphere at 37ºC and 5% CO_2.

(ii) Cell Harvesting

In order to harvest cells, all cell lines were washed twice in phosphate buffered saline (PBS) pH 7.3. Cells were then detached by incubating with 3-5 ml of Trypsin/EDTA (1x) for 5 min at 37ºC. Trypsin was inactivated by adding growth medium and the cell suspension was centrifuged at 1,600 rpm for 5 min. The cell pellet was then resuspended in normal growth medium.

(iii) Cryopreservation & Liquid Nitrogen Cell Recovery

Cells removed from liquid nitrogen were rapidly defrosted and spun down to obtain a cell pellet which was then resuspended in fresh media. Cells were transferred to a 25 cm^2 flasks and incubated. Cells to be stored in liquid nitrogen were prepared as mentioned above and cell pellets were resuspended in 1-2 ml of freezing mix. Cell suspension were transferred to a cryovial and frozen immediately at -70ºC then transferred to liquid nitrogen after 48h.

(iv) Cell Counting

In order to determine the appropriate cell concentration for experimental set up cells were harvested as described above, resuspended in 10 ml of growth medium and counted in a haemocytometer. Cells were then diluted to achieve the correct cell number for each experiment.

(v) Dextran Charcoal Stripping of Foetal Calf Serum

Functional experiments require a less rich medium, free of endogenous stimuli so the effects of growth factors on signalling pathways can be clearly determined. Endogenous steroids were removed from media by double charcoal stripped FCS. After thawing at room temperature, 1L of serum was heat inactivated for 30min at 56ºC. Serum was then incubated with 2000U of type IV sulphatase for 2h at 37ºC and the pH adjusted to 4.2 using 2M HCl. A previously prepared charcoal mix was added to the mix and agitated overnight at 4ºC. The charcoal mix consisted of 5g charcoal and 25mg dextran T70 in 50ml dH_2O which had been stirred overnight. The charcoal was removed via 30min centrifugation at 10,000 rpm (4ºC). The pH was readjusted to 4.2 and a second charcoal mix added to the serum for a further 24h incubation at 4ºC. Serum was centrifuged as previously described with the inclusion of a second spin to remove residual traces of charcoal. The pH was returned to 7.2 with the addition of 2M NaOH. The serum was filtered sterilised and aliquoted for storage at -20ºC.

(vi) Clonal Selection

MCF-7 clones were derived from the original MCF-7 cell line. A cell suspension was diluted and plated onto four 96 well plates. The aim was to have one cell per well so the population would be derived from a single cell. The number of cells in each well was determined by microscopy and wells with more than one cell were discarded. The progress

of the one cell containing wells was monitored and when confluence was reached cells were trypsinised and transferred into a 24 well plate. Cells were transferred into progressively larger cell culture plates, dishes and flasks until there was an adequate cell number. Three clones were obtained referred to as Clones 1, 2 and 3.

2.3.2 Functional Assays

(i) Morphology Studies

Cell lines were seeded in 6-well tissue culture plates at 2-5x10^4 cells per well in 5ml of growth media. Following 24h incubation, cells were washed twice with PBS and media was replaced with DMEM (- phenol red) containing 5% DCSS for a further 24h. The assays are performed in the presence of double charcoal stripped media as it provides a controlled environment so the responses to each individual growth factor are easily observed. Cells were treated with E_2 (1nM) for 48h in quadruplicate wells. Morphology changes were noted by microscopy analysis. Images were taken using a Kodak MDS120 camera.

(ii) Growth Assays

In order to study growth properties of these cells lines the sulforhodamine-B (SRB) colorimetric assay was used. SRB assay as described by Skehan *et al*, 1990 is used for cell density determination based on the measurement of protein content. Cells were seeded into 96-well tissue culture plates. Cells were treated with E_2 (1nM), TGFα (1nM) and a combination of endocrine drugs and inhibitors (Table 2.1). Cell lines were seeded 48h before treatment in phenol-red free DMEM containing 5% DCSS. Plates were treated for 0, 3 and 5 days for each cell line. Treatment was halted by the addition of 50µl of 25% trichloroacetic acid (TCA) to each well for 1h at 4ºC. Plates were then washed 5 times with tap water and allowed to dry. Once plates were dry, 50µl of SRB dye solution was added for 30min at room temperature before washing with 1% glacial acetic acid (4x). Plates were again allowed to dry before resuspending in 150µl of 10mM Tris buffer (pH 10.5) and incubated for 1h at room temperature. Optical densities (OD) for each plate were determined at 540nm using a Biohit BP800 plate reader.

The appropriate cell densities were determined in advance to experimental set up. The MCF-7 cell line was grown in DMEM (+ phenol red) containing 10% FCS. LCC1 and

LCC9 cells were grown in phenol red free DMEM (+ 5% DCSS). The optimal cell density for each cell line was determined taking into account the fact that growth factor treatment would increase cell number (Figure 2.2, 2.3 & 2.4). The relationship between optical density and cell number is considered linear if OD<3 as at higher OD values there is a risk that saturation may be playing a part. Following the results from the seeding density experiments, MCF-7 and LCC1 cell lines were plated at 2500 cells per well whilst LCC9 cells were plated at 1000 cells per well for all the growth studies.

Drugs & Inhibitors	Target	Concentration
Tamoxifen	ERα	1μM
ICI 182,780	ERα	100nM
Herceptin/Trastuzumab	HER2	100nM
2C4/Pertuzumab	HER2	100nM
I-OMe AG538	IGFR	5-10 μM

Table 2.1 Endocrine Drugs & Inhibitors.

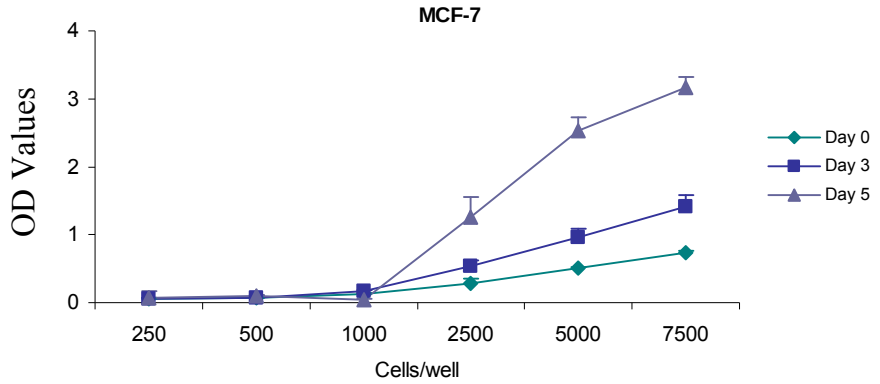


Figure 2.2 Optimum seeding density for SRB assays of MCF-7 cell line. OD values were taken at Day 0, 3 & 5. Each column presents the mean of 6 values. Error bars=SD.

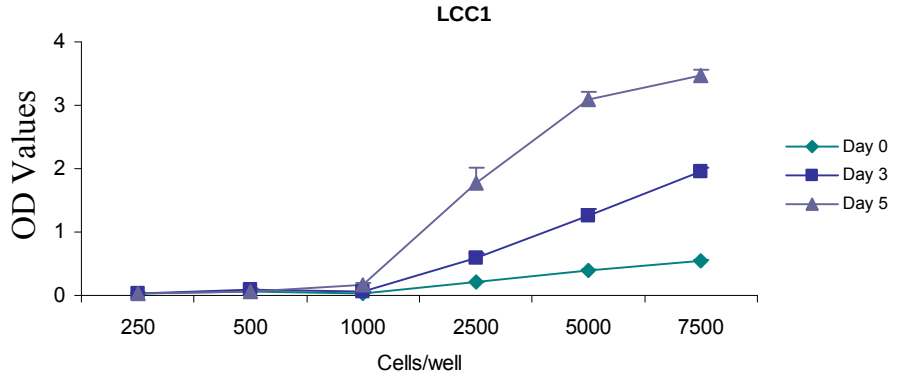


Figure 2.3 Optimum seeding density for SRB assays of LCC1 cell line. OD values were taken at Day 0, 3 & 5. Each column presents the mean of 6 values. Error bars=SD.

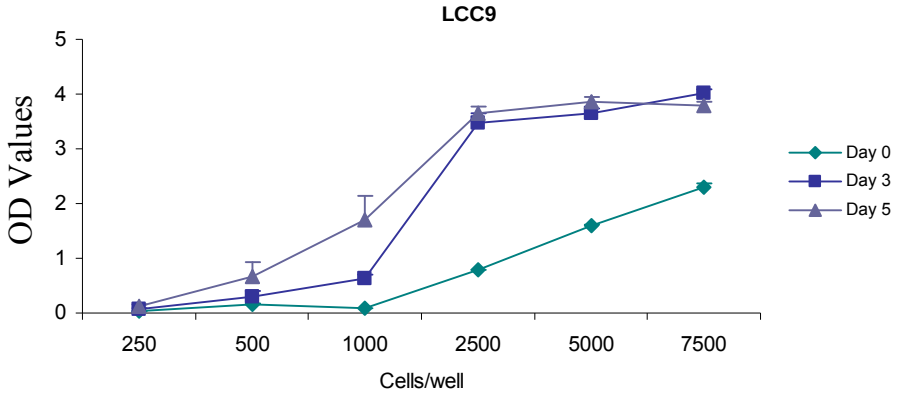


Figure 2.4 Optimum seeding density for SRB assays of LCC9 cell line. OD values were taken at Day 0, 3 & 5. Each column presents the mean of 6 values. Error bars=SD.

2.4 Protein Detection

2.4.1 Protein Extraction & Immunoprecipitation

Cells were harvested as previously described and were seeded into 15 cm (diameter) plates at a density of $1x10^6$ cells per plate for all cell lines. MCF-7 cells were plated in DMEM (+phenol red) containing 10% FCS for 24h at which point the media was replaced with 5% DCSS DMEM (phenol-red free) and grown for a further 48h. Variants cell lines were plated straight into 5% DCSS DMEM and also incubated for 48h. Cells were then treated according to the experiment requirements always in the presence of reduced charcoal stripped media. When cells were ready for protein extraction, plates were washed twice in ice-cold PBS and lysed in ice-cold lysis buffer for 10min in ice. Cells were scraped from culture dishes into eppendorf tubes and debris was cleared by centrifugation at 13,000 rpm for 6min at 4ºC. The amount of protein present in the supernatant was determined using the Bicinchoninic acid assay (BCA) according to manufacturers' guidelines. Samples were prepared into 100µg aliquots and stored at -70 ºC.

Immunoprecipitation detects protein-protein interactions. Briefly, cell lysates are incubated with specific antibodies to the protein of interest. A mix of agarose beads is then added to the solution which binds to the Fc region of the antibody. The proteins bound to the agarose beads are analysed by western blotting. For immunoprecipitation experiments, cells were lysed as described above and a volume of lysate containing 100µg of protein was agitated overnight at 4ºC with 1-10µl of relevant antibody. Following overnight

incubation, protein-G-agarose beads were washed in lysis buffer and 50µl of the bead slurry was added to the lysate/antibody mix. The samples were then incubated for 3 h as before. Beads were washed three times in ice cold lysis buffer. The lysate/antibody/bead slurry mixture was centrifuged for 2min at 2,000 rpm (4ºC) at the end of the three hour incubation and the supernatant was collected using a syringe. 500µl of lysis buffer (minus protease inhibitors) were then added to the solution followed by another spin at 2,000 for 2 min. The supernatant was collected again and the wash was repeated twice more as described. 20µl of 1x loading buffer was added to the bead solution and sample was heated at 95ºC for 5min. Samples were then centrifuged at 13,000 rpm for 1 min and supernatant was removed for loading onto a polyacrylamide gel for Western Blot analysis.

2.4.2 Western Blotting

Cell lysates were prepared and quantified as described. Western blotting is routinely used to detect protein levels. Briefly, proteins are separated on a polyacrylamide gel. Antibodies are used to detect proteins of interest and the results are visualised using chemiluminescence. Aliquots of equal concentration were set up for each protein sample set and their volumes were equalised using lysis buffer. Samples were denatured at 95ºC for 5min in loading buffer and 25-100 µg of protein were used depending on the protein of interest and antibody efficiency. Samples were loaded onto a 7.5-12% polyacrylamide gel (determined by band size or separation required for proteins of interest) which were ran at 80V for 15min and then 200V for 1h. Proteins were then transferred to a permeablised Immobilon-P membrane via a wet transfer method at 30V for 2-3 h. Membranes were then blocked for 1h with 1% blocking agent (diluted in TBS) at room temperature before incubating overnight (4ºC) with primary antibody in 0.5% blocking agent. Following primary antibody incubation, membranes were washed for 5min with TBS-Tween (TBS containing 0.1% Tween-20) three times and then with 0.5% blocking solution before incubating with the appropriate secondary antibody for 1h at room temperature. The final wash before developing consisted of three 5min washes with TBS-Tween followed by another set of three 5 min washes with TBS.

Blots were visualised with either BM chemiluminescence kit or SuperSignal West Femto chemiluminescence substrate and developed using Hyperfilm.

A summary of the antibodies used and the antibody conditions is described in Table 2.2 and 2.3.

Antibodies	Dilution Factor	Source	Supplier Details
EGF Family			
EGFR	1:1000	Rabbit	#2232 Cell Signaling Tech.
Total HER2	1:1000	Rabbit	#2242 Cell Signaling Tech.
Phospho-HER2 (Tyr 1248)	1:1000		#06-229 Upstate
Total HER3 (1B2E)	1:1000	Rabbit	#4754 Cell Signaling Tech.
Phospho-HER3 (Tyr 1289)	1:1000	Rabbit	#4791 Cell Signaling Tech.
Total HER4 (111B2)	1:1000	Rabbit	#4795 Cell Signaling Tech.
Phospho-HER4 (Tyr 1284)	1:1000	Rabbit	#4757 Cell Signaling Tech.
Total IGF-I Receptor β	1:1000	Rabbit	#3027 Cell Signaling Tech.
Phospho-IGF Receptor β (Tyr 1135/Tyr1136)	1:1000	Rabbit	#3024 Cell Signaling Tech.
Akt Pathway			
Total Akt	1:1000	Rabbit	#9272 Cell Signaling Tech.
Phospho-Akt (Ser473)	1:1000	Rabbit	#9271 Cell Signaling Tech.
Phospho-Akt (Thr308)	1:1000	Rabbit	#9275 Cell Signaling Tech.
Akt 1 (2H10)	1:1000	Mouse	#2967 Cell Signaling Tech.
Akt 2	1:1000	Rabbit	#2962 Cell Signaling Tech.
Akt 3	1:1000	Rabbit	#4059 Cell Signaling Tech.
Total PTEN	1:1000	Rabbit	#9552 Cell Signaling Tech.
Phospho-PTEN(Ser 380/Thr382/Thr383)	1:1000	Rabbit	#9554 Cell Signaling Tech.
Total mTOR	1:1000	Rabbit	#2972 Cell Signaling Tech.
Phospho-mTOR (Ser2448)	1:1000	Rabbit	#2971 Cell Signaling Tech.
Total PI3 kinase p85	1:1000	Rabbit	#4292 Cell Signaling Tech.
Phospho-PI3K p85 (Tyr458)	1:1000	Rabbit	#4228 Cell Signaling Tech.
MEK/ERK Pathway			
Total MEK 1/2	1:1000	Rabbit	#9122 Cell Signaling Tech.
Phospho-MEK 1/2 (Ser217/221)	1:1000	Rabbit	#9121 Cell Signaling Tech.
Total ERK 1/2	1:1000	Rabbit	#9102 Cell Signaling Tech.
Phospho-ERK 1/2 (Thr202/Tyr204)	1:1000	Rabbit	#9101 Cell Signaling Tech.
Estrogen Receptor			
ERα (F10)	1:50	Mouse	#sc-8002 Santa Cruz
Phospho-ERα (Ser118)	1:1000	Rabbit	#2515 Cell Signaling Tech.
Phospho-ERα (Ser167)	1:1000	Rabbit	#2514 Cell Signaling Tech.
SRC-1	1:1000	Mouse	#05-522 Upstate
TIF2	1:250	Mouse	#610984 BD Transduction Lab.
AIB1	1:500	Rabbit	#MA1-845 Affinity Bioreagents
PARP	1:1000	Rabbit	#9542 Cell Signalling Tech.
Actin			#CP01

Table 2.2 Primary Antibody List used in Western Blotting and Immunoprecipitation.

Secondary Antibodies	Dilution Factor	Supplier Details
Anti-Rabbit IgG HRP-linked	1:1000	#7074 Cell Signaling Tech.
Anti-Mouse IgG HRP-linked	1:1000	#7076 Cell Signaling Tech.
Anti-Mouse IgM (Goat) Peroxidase Conjugate	1:500	#401221 Calbiochem

Table 2.3 Secondary Antibodies used in Western Blotting.

2.5 RNA extraction & quantitative RT-PCR

2.5.1 RNA Extraction

All cell lines were seeded at $0.5x10^6$ cells per 10cm plate. As described previously for protein detection, MCF-7 cells were plated in DMEM (+phenol red) containing 10% FCS for 24h at which point the media was replaced with 5% DCSS DMEM and grown for a further 48h. Variants cell lines were plated straight into 5% DCSS DMEM and also incubated for 48h. Cells were then treated with the appropriate growth factor/inhibitor.

RNA extraction was performed according to manufacturers' instructions (Absolutely RNA Miniprep Kit, Stratagene). Briefly, 600µl of lysis buffer (+ β-Mercaptoethanol) was added to each plate, allowed to mix and incubated for a couple of minutes. RNA was then isolated using a RNA binding spin cup. The samples were then DNase treated (DNase digestion buffer & reconstituted RNase-Free DNase I) for 15min at 37ºC. After this incubation, the spin cups were washed with low-salt and high salt wash buffers and the RNA was then eluted in 50µl of elution buffer.

RNA was quantified using the Nanodrop spectrophotometer.

2.5.2 Quantitative RT-PCR

QuantiTect SYBR Green System was used for performing one step RT-PCR according to manufacturers' instructions. Briefly, RT-PCR allows mRNA to be amplified. Firstly, the mRNA is reverse transcribed into cDNA followed by PCR amplification cycles. The amount of product was detected using Sybr Green dye which binds to double stranded nucleic acids (Figure 2.5). Total reaction volume of 15µl: 7.5µl 2xQuantiTect SYBR Green Master Mix; 0.375µl primer mix (20µM); 0.15µl RT Mix; 2.975 RNase-free water and 4µl of RNA. Real time cycler conditions were RT: 50ºC for 30 min; PCR: initial activation 95ºC for 15 min followed by 40 cycles of denaturation 94ºC for 15 sec, annealing 57ºC for 30 sec, extension 72ºC for 30 sec, and a final extension of 72ºC for 60 sec.

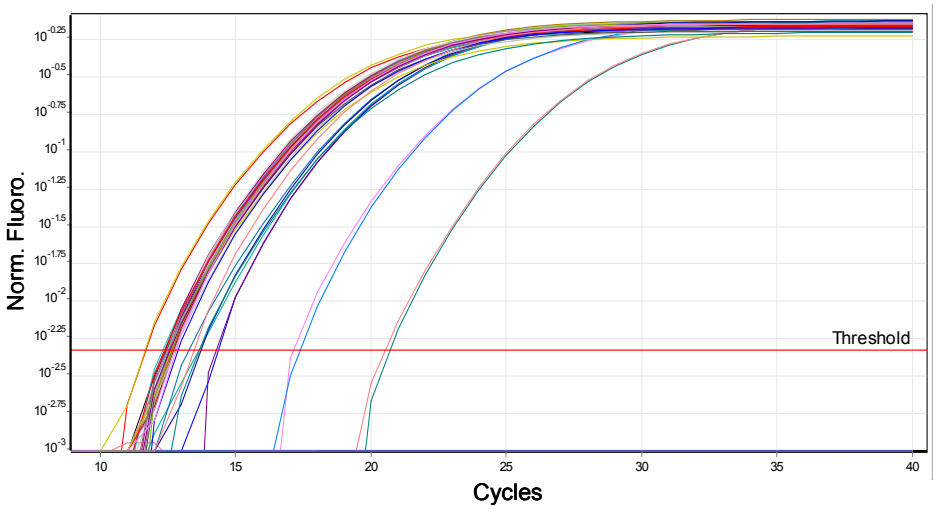


Figure 2.5 Typical Real Time RT-PCR.amplification. The figure shows the amount of fluorescence generated by Sybr Green versus the number of PCR cycles. The samples with the most amount of mRNA can be found to the left of the graph. A greater number of specific mRNA results in amplification at a faster rate found at an earlier cycle.

EGF Family	
EGFR	fwd TGCACTCAGAGAGCTCAGGA\ rev CAGCGCTACCTTGT
HER2	fwd CTGAATGGGTCGCTTTTGTT\ rev CTCGTTGGAAGAGGAACAGC
HER3	fwd CTCCTTTGTGCACAGTTCCA\ rev GCGGCACTTTTCTCTACTGG
HER4	fwd GGAAATTGGAGCAGGTGTGT\ rev GCGGCACTTTTCTCTACTGG
IGFR	fwd GTTGGGAAGGGGATCATTTT\ rev ATGAAAACCATTGGCTGTG
Akt Signalling	
Akt 1	fwd ACCAGGTATTTTGATGAGGAGTTA\ revCGCTGTCCACACACTCCAT
Akt 2	fwd ATGCTGGCCGAGTAGGAGAA\ rev GCCCAGTCCATCACAATC
Akt 3	fwd AGGACCGCACACGTTTCTAT\ rev TTCTGGAGTGCCACAGAATG
PTEN	fwd GGACGAACTGGTGTAATGATAT\ revCTACTGTTTTTGTGAAGTACAG
mTOR	fwd CCAACAGTTCACCCTCAGGT\ rev CTGCCACTCTCCAAGTTTC
PIK3CA	fwd CCCCTCCATCAACTTCTTCA\ rev GGTTGCCTACTGGTTCAAT
Estrogen Pathway	
ERα	fwd CCACCAACCAGTGCACCATT\ rev GTCTTTCCGTATCCCACCTTTC
SRC-1	fwd CATGCTTATGAGGCAGCAAA\ rev ATTCCAGTGCCAAACTGTCC
TIF2	fwd AGCCTGTGAGAGGGCTGTTA\ rev AATGAGAGAGGGGAAGGGAA
AIB1	fwd CCCTTTTATCTACTCTGTCATC\ rev CCAGATGTAGAGGAGGAGAC
pS2	fwd TTGTGGTTTTCCTGGTGTCA\ rev CCGAGCTCTGGGACTAATCA
B-Actin	fwd GATGGAGCCGCCATCCACACGG\ rev CTACGTCGCCCTGGACTTCG

Table 2.4 Primer Sequences used in Real Time RT-PCR.

2.6 Multiplexed Assays for *PIK3CA* Mutations

The assay used here was based on a paper published by Board et al (2008) that combines two techniques for detection mutations: Amplification Refractory Mutation System (ARMS, Astrazeneca) and Scorpions (DxS). Together they provide a sensitive assay for the detection of *PIK3CA* mutations. The ARMS assay relies on the principle that extension only occurs when the 3'terminal base of a primer matches its target (Figure 2.6). Scorpion probes combine a PCR amplification primer and a probe which recognises the amplicon generated during the PCR reaction (Figure 2.6). Binding of probe to the target (amplicon-specific sequence) produces a fluorescent signal, the strength of which is proportional to the amount of amplicon. Primer sequences described in Table 2.5. Scorpion primers were labelled with different fluorophores to allow multiplex mutational analysis.

Cells were harvested as previously described and counted. Pellets containing $1x10^6$ cells were then prepared and stored at -70ºC. DNA was extracted using the QIAamp DNA FFPE Kit (Qiagen) according to manufacturers' instructions. Briefly, cell pellets were resuspended in 180µl of ATL buffer and incubated with proteinase K for 1h at 56ºC. Proteinase action was inactivated by heating at 90ºC for 1h. Samples were then spun at full speed for 2min and 150µl were transferred to new tubes before adding 300 µl of AL buffer /ethanol mix. Samples were loaded to a QIAamp MinElute column and centrifuged at 8000rpm for 1min. The filtrate was discarded and the column was washed twice, first with AW1 buffer and then with AW2 buffer centrifuging in between washes. DNA was eluted from the column by incubating the dry membrane with 30µl of ATE buffer for 5min at room temperature. Each column was centrifuged at full speed for 1min to elute the DNA.

ARMS Primers	
Arms Control Primers	5'-AGATGATCTCATTGTTCTGAAACAG-3'
H1047R	5'-TGTTGTCCAGCCACCATGCC-3'
H1047L	5'-TGTTGTCCAGCCACCATGCA-3'
E542K	5'-CTTTCTCCTGCTCAGTGATTCT-3'
E545K	5'-ACTCCATAGAAAATCTTTCTCCTGATT-3'
Scorpion Primers	
Scorpion Control Primers	Rox-CCGGCCAATTCAACCACAGTGGCCGG-que-heg-GGCTTGAAGAGTGTCGAATTA
Exon 20	Fam-CGCGGCATGAAATACTCCAAAGCCGCG-que-heg-CCTAGCCTTAGATAAAACTGAGCAA
Exon 9	Hex-CGCGCTCGTGTAGAAATTGCTTTGAGCGCG-que-heg-AATGAATTAAGGGAAAATGACA

Table 2.5 Primer Sequences used in the Multiplex assay.

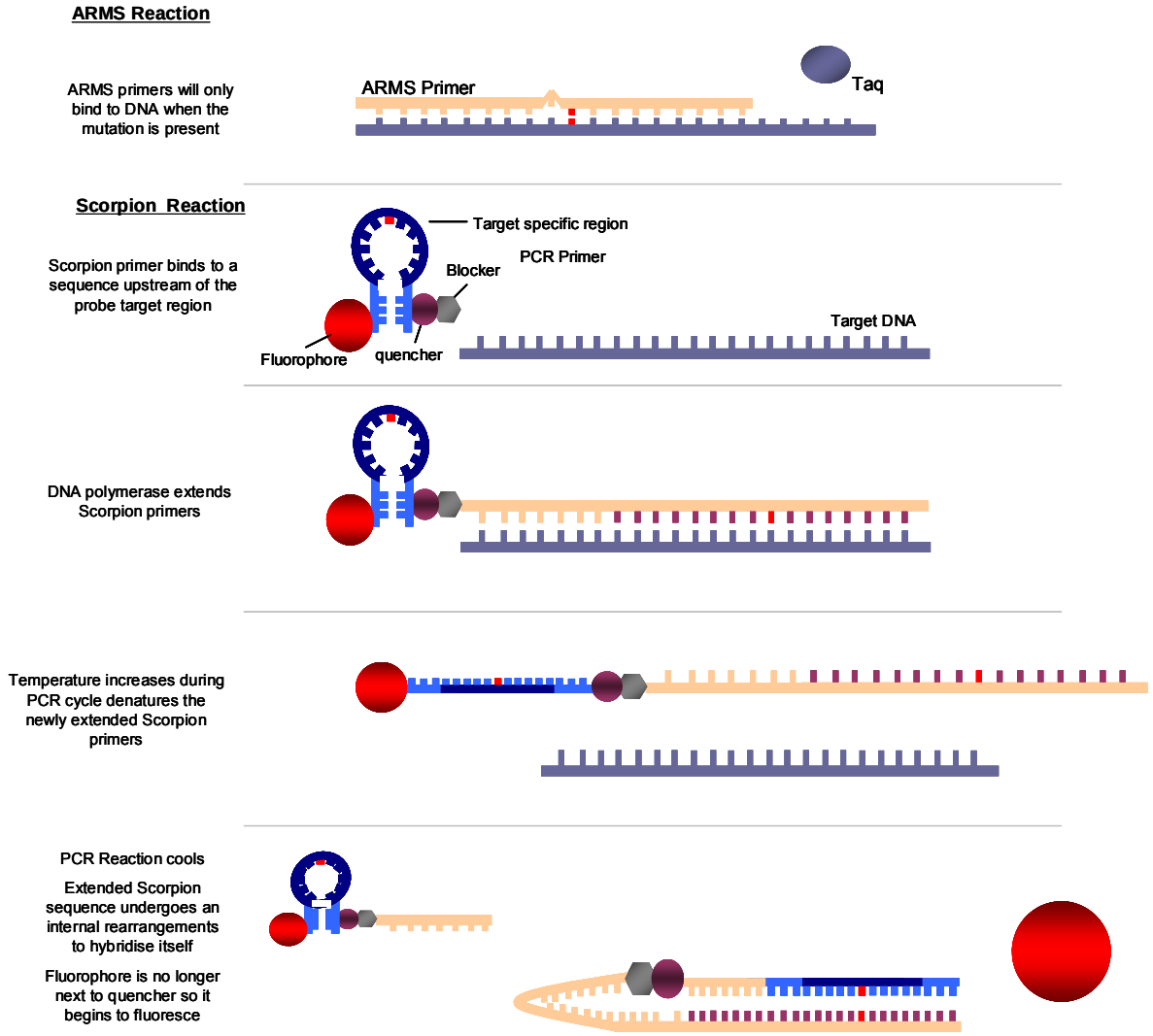


Figure 2.6 Overview of multiplex assay. The assay combines ARMS and Scorpion primers which are very specific in identifying mutations and produce a measurable fluorescence signal, respectively. Together they provide a sensitive assay.

Total reaction volume of 25µl comprises of: 2.5µl of 1x HotGoldStar PCR Buffer; 2µl of 4mM $MgCl_2$; 1µl of 0.2mM dNTP; 1.25µl of 0.25 µM Control Scorpion; 1.25µl of 0.25 µM Exon 20 Scorpion; 1.25µl of 0.25 µM Exon 9 Scorpion; 0.6µl of 0.12U/µl; 1.25µl of 0.25 µM ARMS primers (H1047R and E542K) **OR** 1.25µl of 0.25 µM ARMS primers (H1047L and E545K). PCR conditions were: 95ºC for 10min followed by 45 cycles of 90ºC for 30secs and 60ºC 60secs. Detection of different fluorescence using three different

channels: FAM/Sybr, JOE and ROX. FAM-containing Scorpion primers detected mutations on exon 20 whilst Hex-containing Scorpion primers (JOE channel) detected on exon 9. A typical amplification plot is shown in Figure 2.7.

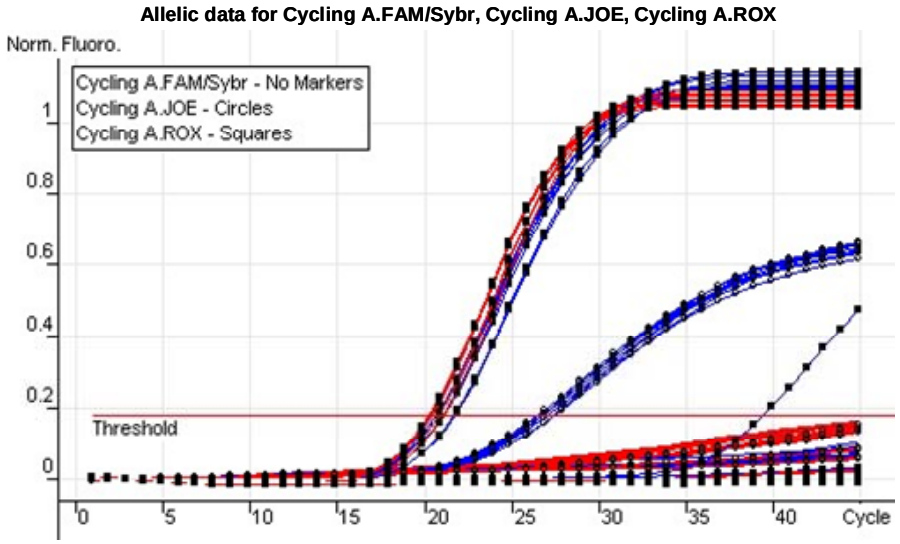


Figure 2.7 Amplification plot for Multiplex analysis for *PIK3CA* mutations. FAM/Sybr and JOE channels detect mutations in exons 20 and 9, respectively. The ROX channel detects the control Scorpion primers.

2.7 Flow cytometric Analysis of Cell Cycle

Flow cytomety was used to study cell cycle. Flow cytometry requires cells to be suspended in a stream of fluid with a laser light directed into it. A number of detectors convert the light signals into electronic signals. In this case, flow cytometry was used to detect DNA content in these cells. Cells were prepared as previously described and $1x10^6$ cells were plated into 10cm plates. Treatments were administered for 3 and 5 days. Cells were trypsinised as before taking extra care not to lyse cells. Trypsin was neutralised by adding 2ml of 5% DCSS DMEM (-phenol red) media and the samples were transferred to FACS tubes before centrifugation at 17,000rpm for 4 min. The pellets were then resuspended in 1ml of ice cold PBS and centrifuged again as before. The resulting pellets were this time resuspended in 200µl of Vindelovs Citrate buffer and stored at -20ºC. At the time of analysis, samples were thawed at room temperature before adding 450µl per tube of Solution A. The samples were briefly mixed and incubated for 3min. The samples were then neutrralised with the addition of 375µl of Solution B for 10min. Finally, 250µl of

Solution C was added per tube and incubated on ice for a further 10min. Samples were immediately analysed in FACScalibur (Becton Dickinson) and the data was obtained with the CellQuest 1.2.2 programme. Results were further analysed using ModFit LT 1.01 software (Figure 2.8).

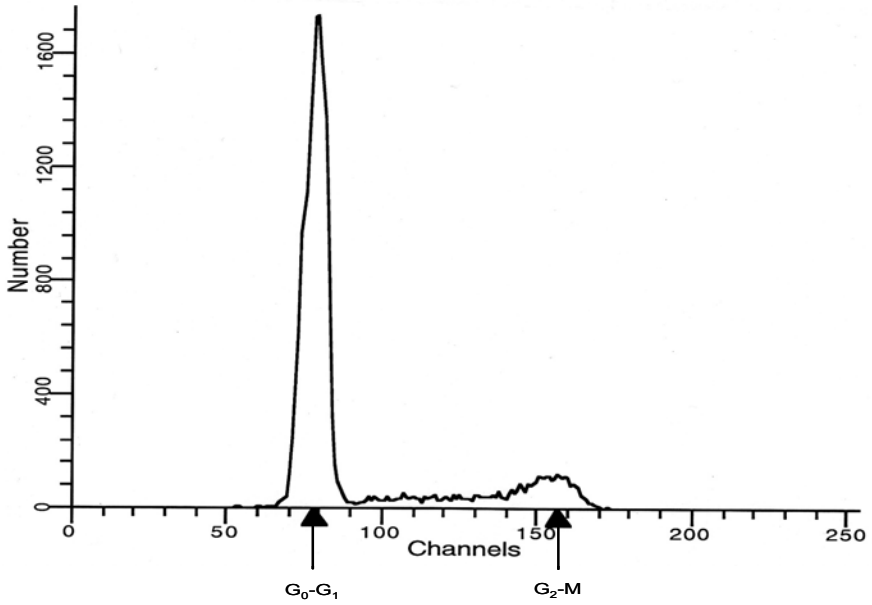


Figure 2.8 Typical cell cycle profile generated using ModFit LT 1.01 Software.

2.8 RNAi Studies

siRNA transfections as described below were used in p160 cofactor studies (Chapter 4) and Akt isoform phosphorylation levels (Chapter 6).

All cell lines were plated at $0.5x10^6$ cells per 10cm plate. MCF-7 cells were plated in phenol red DMEM which was replaced with 5% DCSS DMEM (-phenol red) 24h later as previously described. LCC1 and LCC9 cells were seeded directly into phenol red free DMEM supplemented with 5% DCSS for 24h. Oligofectamine reagent (Invitrogen) was firstly diluted in Opti-MEM (#31985-070; Gibco) reduced serum media before adding the appropriate siRNA. This mixture was incubated at 37ºC for 15min. Cells were then transfected for 4 h. Oestrogen was then added if required and incubated for a further 48h before RNA and protein extractions performed as previously described. siRNA sequences (100nm) were as follows: Negative siRNA (Upstate M-003401); AIB1 (Qiagen #1024591); SRC-1 (Qiagen #1024927); SRC-2: 5'AAGTCAGATGTATCCTCTACA (Qiagen); ERα: 5'AAA-CAGGAGGAAGAGCTGCCA (40 nmol; Ambion, Cambridgeshire).

Akt RNAis (2.5µl) were diluted in 250µl of Opti-MEM in 6-well plates. 7.5µl of Lipofectamine RNAiMAX (Invitrogen) was then added to each well, mixed gently and incubated for 10-20min at room temperature. Cells were harvested as previously described and all cell lines were diluted to a concentration of $1.7x10^5$ cells/ml in 5% DCSS DMEM (-phenol red). 1.5ml of the cell dilution was added to the RNAi-lipofectamine complexes and incubated for 48h before RNA collection. For protein extraction plates were incubated for 72h. siRNAs were used at a concentration of 50nM from a stock solution of 20nM. siRNA sequences were as follows: Akt 1: 5'ACCTGACCAAGATGACAG; Akt 2: 5' AAGTGGGTCCGCTGGT; Akt 3: 5'AGGAGGTACAAGCTTTTTA (Applied Biosystems UK)

2.9 Migration & Invasion Assays

2.9.1 Migration Assays

In order to assess migration ability in these cell lines a Quantitative Cell Migration Assay Kit (Chemicon) was used. Three different kits were used as they were coated with different matrix proteins, collagen I, fibronectin and vitronectin. Experiments were performed according to manufacturers' instructions. Briefly, this assay uses a system of Boyden chambers coated with specific matrix proteins to measure cell migration. Cells were plated at a concentration of $2.5x10^6$ cells per well. Each cell line was plated in phenol red free DMEM supplemented with 5% DCSS in duplicate. There are two plates as part of the kit, one Test plate and a Control plate. The Test plate contains boyden chambers coated with the matrix protein (Collagen I, Fibronectin & Vitronectin). The Control plate, on the other hand, contains a row of matrix protein coated well and a row of BSA coated wells. The former serves as a adhesion control whilst the latter provides a control for migration. Plates were incubated for 24h in a tissue culture incubator. Each Boyden chamber was cleaned taking extra care not to touch the underside of the chamber as it contains the migratory cells. Boyden chambers were then stained with a crystal violet stain (400µl) for 30min at room temperature before washing in PBS three times. The clean chambers were then placed into extraction buffer (300µl) and shaken for 10min in order to elute stain. 100µl of the stained solution was transferred onto a clean microtitre plate and the absorbance was read at 560nm.

Cell migration was illustrated graphically by a "bar" chart by comparing migration in BSA-coated chambers with migration in matrix coated chambers. Cell migration in BSA-coated chambers is usually very low due to the lack of stimulus thus these chambers are used as a blank measuring the amount of background staining.

2.9.2 Invasion Assays

Similarly to the migration assays, the Cell Invasion Assay Kit (Chemicon) used in these studies relies on a Boyden chamber system. Each 24-well tissue culture contained an insert coated with a thin layer of dried extracellular matrix (ECM) over a polycarbonate membrane (8μm pore size). Invading cells are able to travel through the ECM layer and attach to the bottom of the polycarbonate membrane. The kit was used according to manufacturers' instructions. Briefly, plates were rehydrated for 2 h by adding reduced serum media (5%DCSS DMEM) to the interior of the inserts. After 2h, the media was removed and 500μl of cell suspension (in 5% DCSS DMEM) was added to each well ($0.5x10^6$ cells/ml). Media containing 10% foetal bovine serum was added to the lower chamber to act as a chemoattractant. Samples were loaded in quadruplicate. The plates were incubated for 48h in a tissue culture incubator. The chambers were then cleaned in order to remove any non-invading cells found in the insert, particularly in the interior of the inserts. The invading cells found on the lower surface of the inserts were stained for 20 min at room temperature with crystal violet stain. After staining the inserts were washed in distilled water several times and allowed to dry. The crystal violet stain stain was dissolved in 10% acetic acid (150μl) and 100μl of the stained solution was transferred to a clean 96-well plate. Absorbance was read at 560nm.

2.10 Immunohistochemistry

(i) Patients

The breast tumour material used here has been previously used in a number of studies and was kindly donated by Professor John Bartlett (Edinburgh Cancer Research Centre, University of Edinburgh). It comprises 402 ERα-positive breast carcinomas belonging to patients diagnosed between 1983 and 1999 at Glasgow Royal Infirmary. ERα positivity had been previously determined and defined as 10% or more positive tumour cells staining. This is in accordance to current local pathology guidelines. Patients were treated with

adjuvant tamoxifen for an average of 5 years. Ethical approval was obtained from the local ethics committee.

(ii) SRC-1 Immunohistochemistry

Immunohistochemistry for SRC-1 (#2191, Cell Signalling) was performed on previously prepared tissue microarrays using a standard immunoperoxidase procedure. Sections were dewaxed and rehydrated before proceeding with antigen retrieval which was done by microwaving the slides under pressure for 10min in Citrate Buffer. The slides were then treated with 3% H_2O_2 for 10min in a stirrer in order to quench peroxidase activity before incubating in serum free block solution (Dako) for 20min to eliminate any unspecific background staining. Slides were incubated with primary antibody (1:600 dilution) overnight at 4ºC. The sections were washed twice in TBS-Tween for 5min. EnVision (Dako) was used for signal amplification and positive staining was detected by incubating with 3,3'diaminobenzidine solution (DAB, Vector Laboratories). Finally, the sections were counterstained with hematoxylin, dehydrated and mounted.

(iii) Scoring of IHC Results

Slides were digitized using Ariol, a commercially available image analysis system (Applied Imaging Inc., San Jose, California). Scores from 0 (Negative) to 3+ (strongly positive) were automatically generated by the Ariol image analysis software for each core based on the percentage of positive cells and the intensity of signal. The tumour tissue in each individual core was manually selected before automated scoring.

(iv) Statistical Analysis

The statistical package SPSS (version 9.0) was used for all the IHC statistical analysis. The Kaplan-Meier life tables were plotted to assess overall survival and disease-free survival.

Chapter 3

Characterisation of Breast Cancer Cell Lines

The three stage MCF-7 cell line model was used in this study as it mimics acquired endocrine resistance often observed in the clinical setting. LCC1 cells acquired oestrogen independence however remain anti-oestrogen sensitive whilst LCC9 cells are both oestrogen independent and endocrine resistant. MCF-7 and its derivative cell lines LCC1 and LCC9 were first characterised to establish whether cellular processes such as growth, apoptosis, cell cycle and cell migration/invasion may be altered in the advent of endocrine resistance.

3.1 Growth Characterisation and Morphology

The wild type MCF-7 breast cancer cell line is oestrogen dependent and is sensitive to anti-oestrogens such as Tamoxifen and ICI 182,780. This is confirmed in Figure 3.1. Oestrogen (Fig. 3.1a) and TGFα (Fig. 3.1b) stimulate growth (twofold increase) in MCF-7 cells although oestrogen appears to be more effective. Tamoxifen reduces growth in the presence of oestrogen (Fig. 3.1a) but does not affect TGFα driven growth (Fig. 3.1b). In the absence of oestrogen or growth factor stimulus, growth of MCF-7 cells is negligible and the cells are said to be in a static state. Treatment with the pure anti-oestrogen ICI completely reverses oestrogen and TGFα stimulation in MCF-7 cells (Fig. 3.1).

LCC1 cells are oestrogen independent as a result of growing in a low oestrogen environment but retain a certain level of oestrogen responsiveness. This is evident in Figure 3.1a. The effects of TGFα, on the other hand, appear to be minimal as the growth curve mimics that of control (Fig. 3.1b). Like the parental MCF-7 cell line, LCC1 are still responsive to the anti-estrogenic effects of tamoxifen and ICI 182,780 (Fig 3.1).

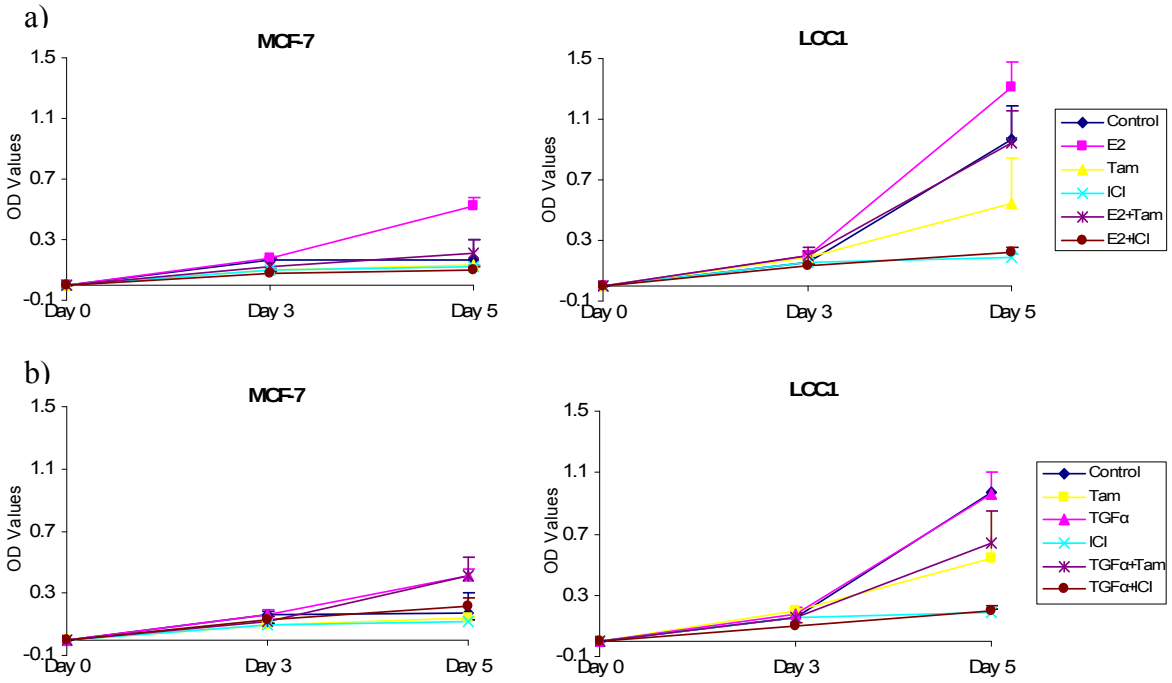


Figure 3.1 Growth characterisation of MCF-7 & LCC1 cells. Cells were treated in the presence of growth stimulatory factors E_2 **(a)** and TGFα **(b)**. Cell OD values were measured at Day 0, 3 & 5. Data plotted represent means of six OD values. Error Bars=SD.

In contrast to MCF-7 and LCC1 cells, LCC9 cells are completely oestrogen independent and unresponsive to oestrogen as indicated by the fact that untreated and oestrogen treated LCC9 cells have comparable growth effects (Fig. 3.2a). TGFα treatment also appears to have little effect on growth in this cell line (Fig 3.2b). Moreover, LCC9 cells are fully resistant to tamoxifen and ICI 182, 780 as their growth fails to be inhibited by drug treatment (Figure 3.2).

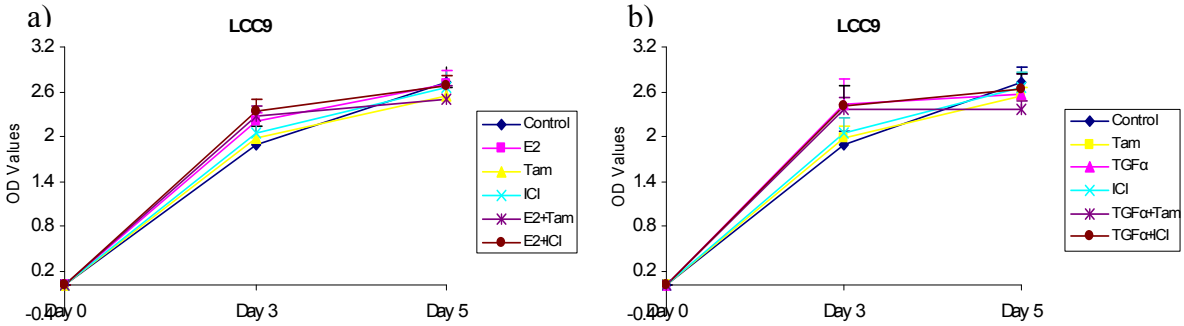


Figure 3.2 Growth characterisation of LCC9 cells. Cells were treated in the presence of growth stimulatory factors E_2 **(a)** and TGFα **(b)**. Cell OD values were measured at Day 0, 3 & 5. Data plotted represent means of six OD values. Error Bars=SD.

Cellular morphology was another of the characteristics analysed to determine whether there are differences between endocrine sensitive and resistant cell lines. All cell lines were grown for 48 hours in charcoal-stripped serum and then treated with oestrogen.

All three cell lines grow as monolayers although MCF-7 cells seem to distribute more evenly cross the surface whilst LCC1 and LCC9 cells appear to prefer to grow in clusters (Figure 3.3). Untreated MCF-7 cellular morphology is quite distinct creating what is often referred to as a "cobblestone" effect (Figure 3.3). In the presence of oestrogen, the appearance of MCF-7 cells changes to form clusters similar to those of LCC1 and LCC9 cells under control conditions (Figures 3.4 & 3.5). Despite having little effect on growth rates, oestrogen did alter the morphology of LCC1 and LCC9 cells as the clusters appear to become more pronounced (Figures 3.4 & 3.5).

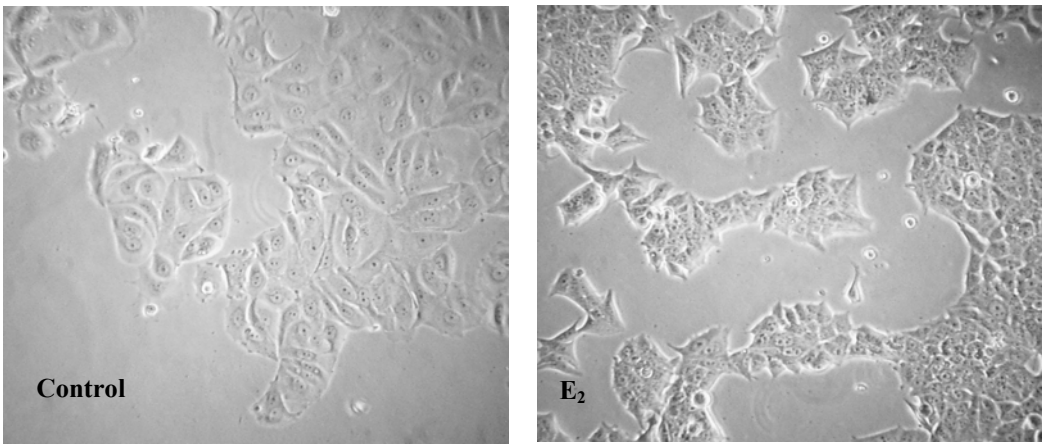


Figure 3.3 Cellular morphology of MCF-7 cell line. Cells were grown in stripped serum in the absence or presence of E_2 for 48h. Magnitude x100.

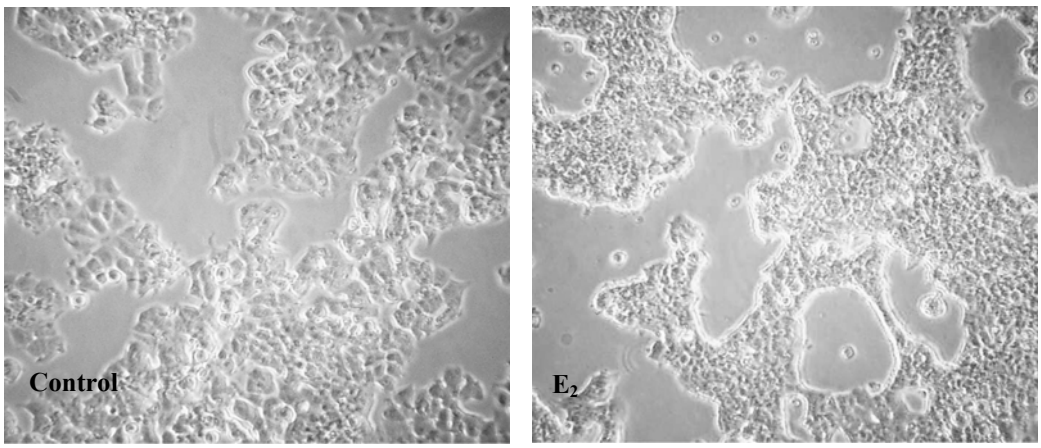


Figure 3.4 Cellular morphology of LCC1 cell line. Cells were grown in stripped serum in the absence or presence of E_2 for 48h. Magnitude x100.

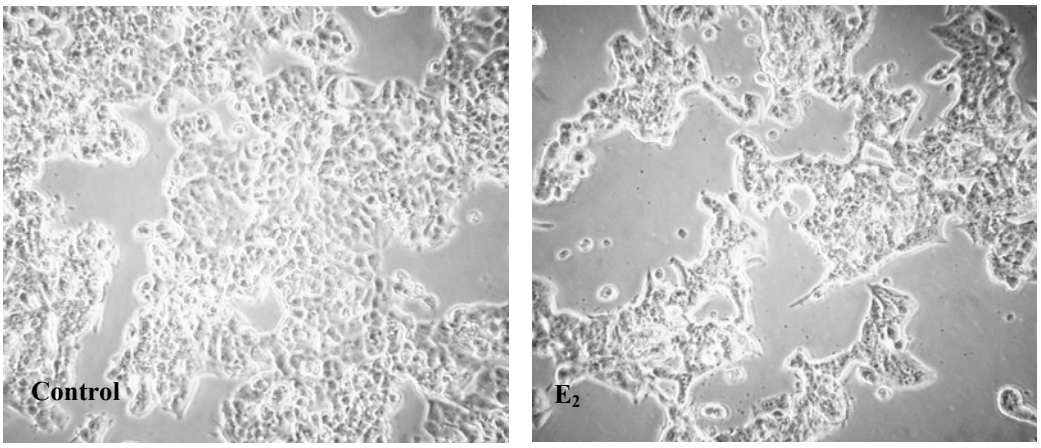


Figure 3.5 Cellular morphology of LCC9 cell line. Cells were grown in stripped serum in the absence or presence of E_2 for 48h. Magnitude x100.

3.2 Clonal Selection

It became evident during cell cycle experiments that MCF-7 cell line culture appeared to contain two different populations. A typical cell cycle profile would consist of two peaks representative of cells at the G_0-G_1 and G_2-M phases. Figure 3.6 shows the profile obtained which present two peaks at the G_0-G_1 phases suggesting there are two cell populations present. This analysis does not however tell us the origin of this second cell population but it is possible that routine cell culture may have given rise to a secondary MCF-7 cell population.

MCF-7 cells are the most routinely used breast cancer cell line and its popularity has resulted in the production of a number of different cellular stocks. To eliminate the risk of using a different subline of MCF-7 from the one routinely used in our lab, clonal selection was used to select clones from the MCF-7 stock with the suspected clonal variation (Figure 3.7). The process began with a single cell per well (96 well plates) isolated by serial dilution hence it was assured that the population obtained was derived from a single cell.

Three MCF-7 clones were collected and fully characterised to determine how they compared with the parental wild-type cell line. Furthermore, characterising each of the clones would establish which clone was the most appropriate clone to use in future experiments.

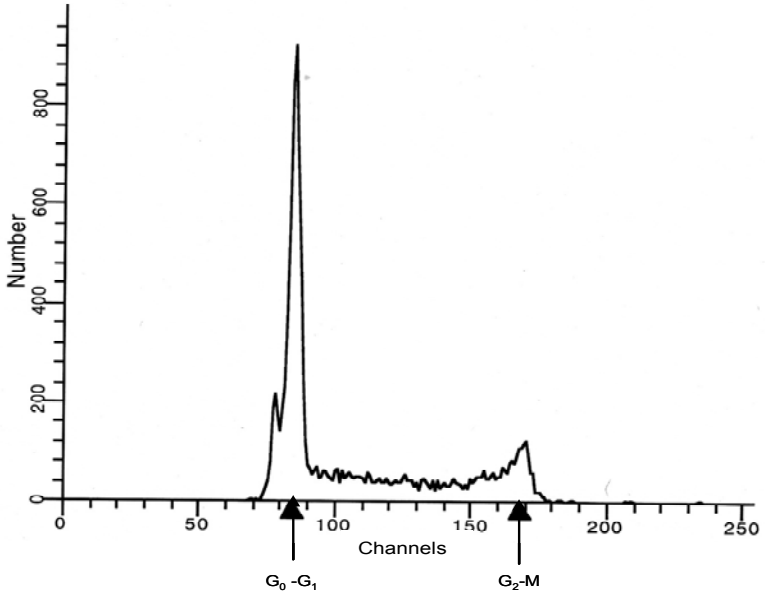


Figure 3.6 Cell Cycle Analysis of MCF-7 cells. Figure representative of triplicate samples. Note the two peaks at G_0-G_1 and G_2-M phases suggesting there are two populations of cells.

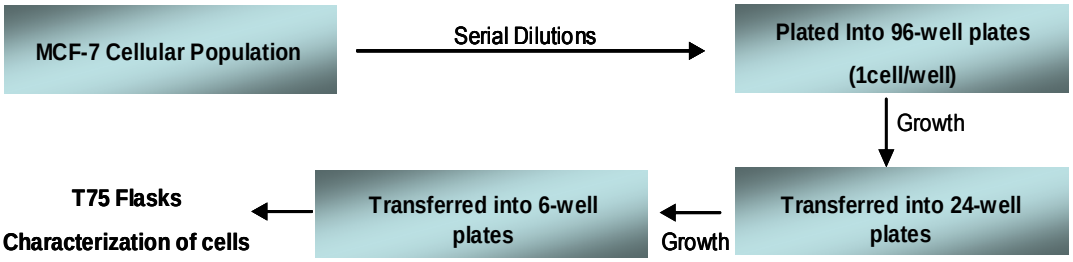


Figure 3.7 Diagram of Clonal Selection Procedure.

The MCF-7 clones were first analysed by flow cytometry (Figure 3.8). This was to determine whether each clone contained one or more populations as previously observed in MCF-7 cells. This was not found to be the case in the MCF-7 clones as can be observed by the single peak representing cells at the G_0-G_1 phase and a second peak for cells in G_2-M phase (Figure 3.8). It can therefore be concluded that, at least at the cell cycle level, MCF-7 clones consist of a single population.

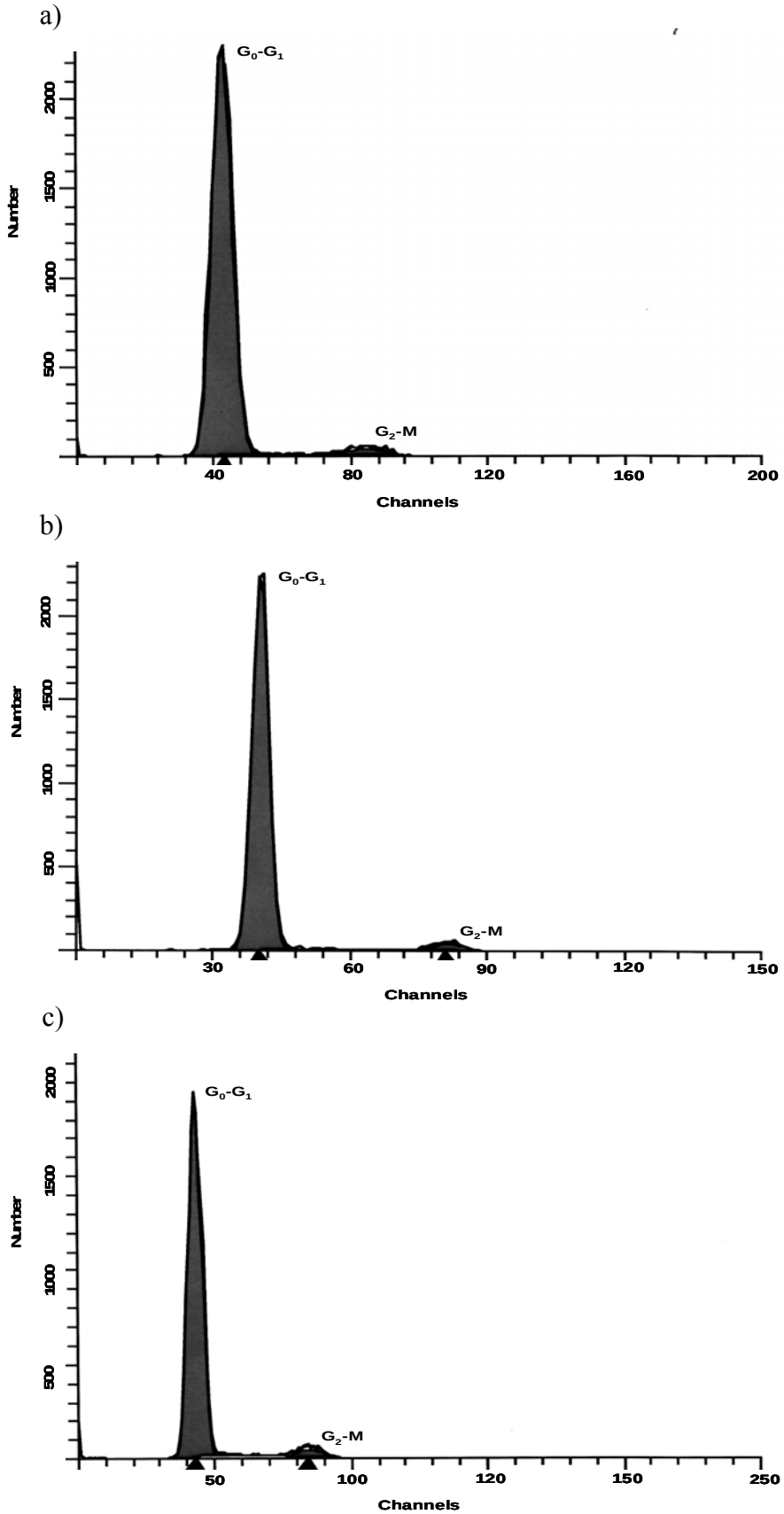


Figure 3.8 Cell Cycle analysis of MCF-7 Clones: Clone 1 **(a)**, Clone 2 **(b)** & Clone 3 **(c).** Figure representative of triplicate samples. This was a simple method to detect whether clonal selection was successful in eliminating a mixed population.

3.2.1 Growth Characterisation

All three MCF-7 clones were static under control condition and require oestrogen or growth factor stimulation (e.g. TGFα) as previously described is the case in MCF-7 cells (Figures 3.9 & 3.10). The responses to tamoxifen were variable in the clones. Although growth is certainly reduced by tamoxifen in the presence of oestrogen in all three clones, the extent of response is different (Figures 3.9 & 3.10). This probably reflects the agonist effects of tamoxifen

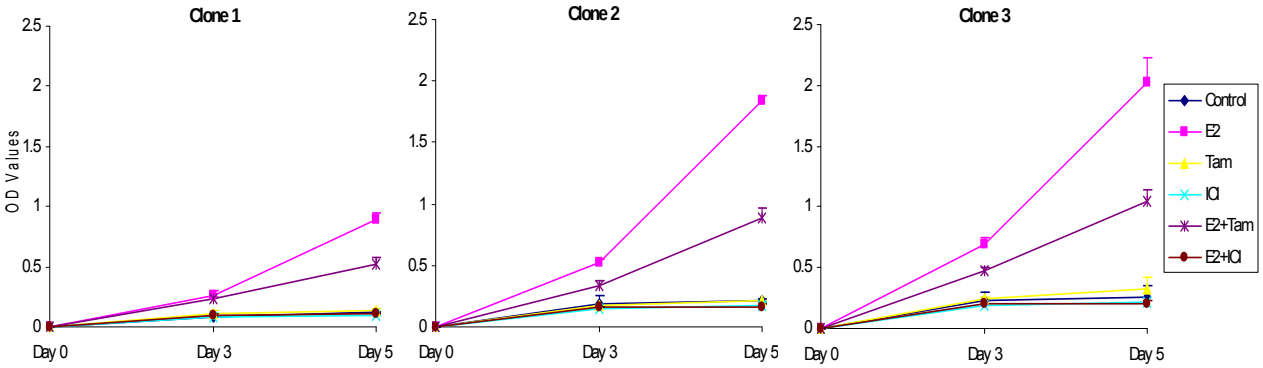


Figure 3.9 Growth characterisation of MCF-7 Clone 1, 2 & 3. Cells were treated in the presence of growth stimulatory factor E_2. Cell OD values were measured at Day 0, 3 & 5. Data plotted represent means of six OD values. Error Bars=SD.

ICI 182,780 effects on MCF-7 clones are also consistent with previous studies. In all clones ICI 182,780 completely reverses the growth stimulatory action of oestrogen and reduced growth to control levels (Figures 3.9 & 3.10). According to this data, growth responses to oestrogen and anti-oestrogens in MCF-7 clones are comparable to the previously shown responses of MCF-7 cells.

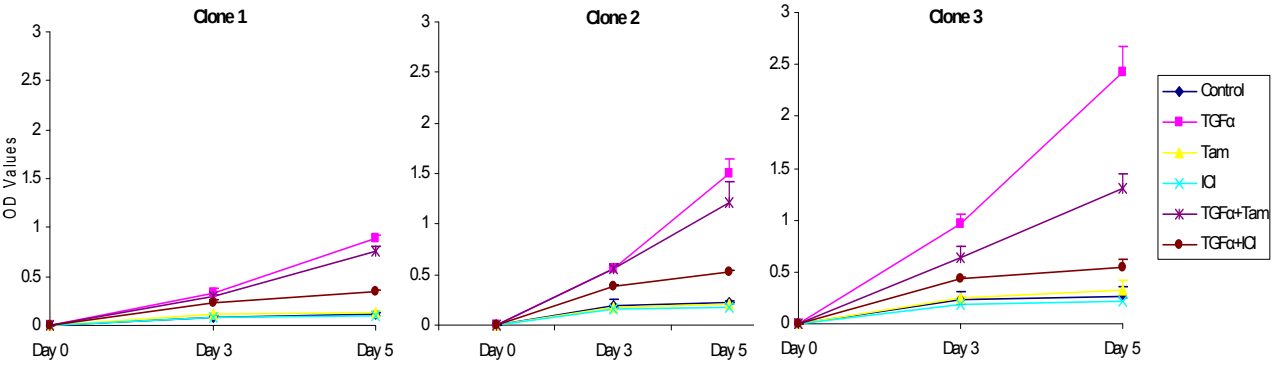


Figure 3.10 Growth characterisation of MCF-7 Clone 1, 2 & 3. Cells were treated in the presence of growth stimulatory factor TGFα. Cell OD values were measured at Day 0, 3 & 5. Data plotted represent means of six OD values. Error Bars=SD.

3.2.2 General Morphology

The morphology of the three MCF-7 clones was similar to that of MCF-7 (Figure 3.11 a, b & c). Interestingly, MCF-7 clone 3 appears to grow at a much slower rate than Clone 1 and 2. This may reflect an actual difference between the clones. The phenotypic response to oestrogen also appears to be the same as previously observed. All three clones form a "cobblestone" effect which changes to form clusters following oestrogen treatment, an appearance very similar to that of LCC1 and LCC9 (Figure 3.11 a, b & c).

Growth curves and phenotypic analyses indicate that the MCF-7 clones have properties consistent with those of MCF-7 cells.

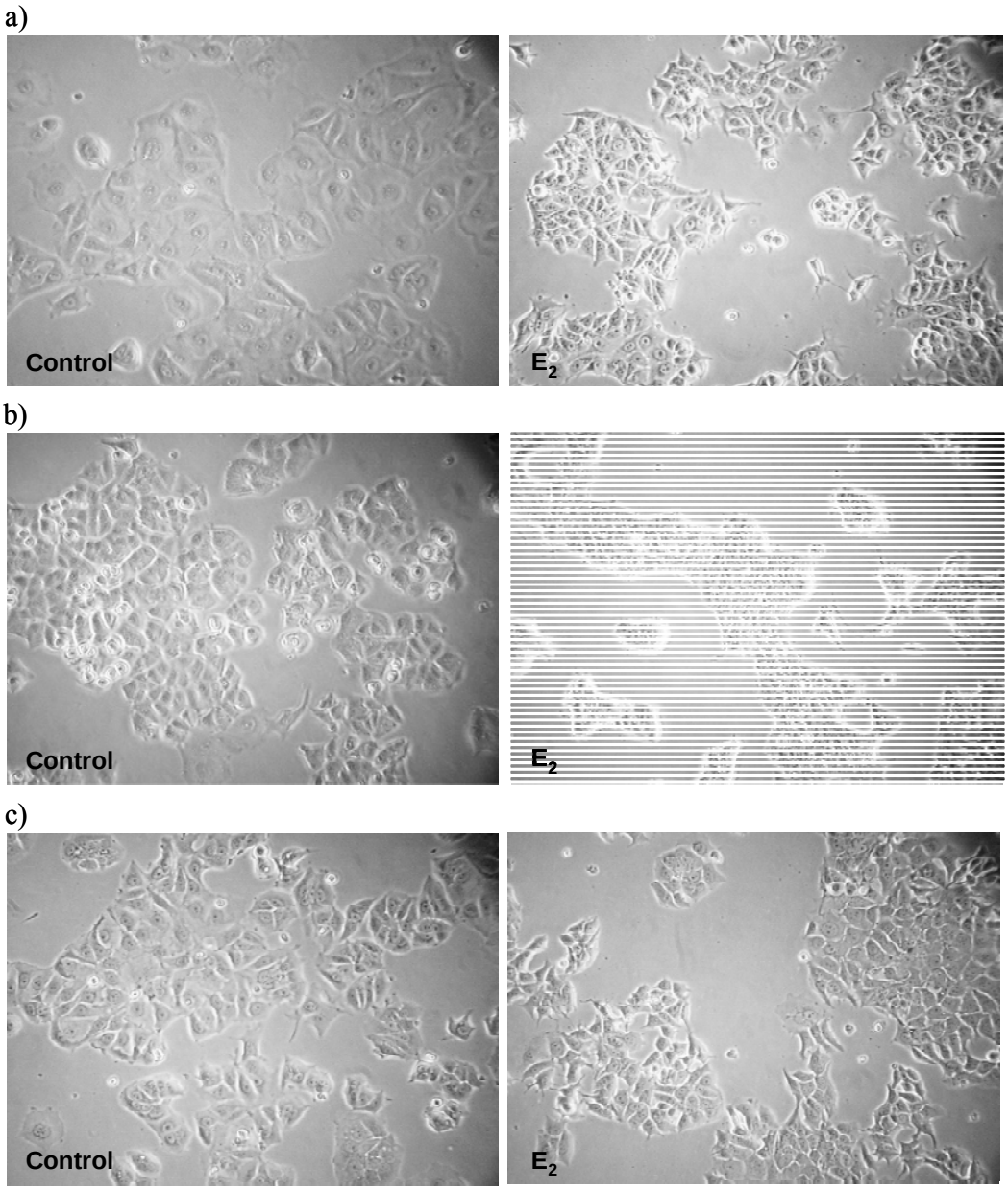


Figure 3.11 Cellular Morphology of MCF-7 Clone 1 (a), Clone 2 (b) & Clone 3 (c). Cells were grown in stripped serum in the absence or presence of E_2 for 48h. Magnitude x100.

3.2.3 Expression Profiling

3.2.3.1 Signalling Proteins

MCF-7 cells are an oestrogen receptor positive cell line so it was important to determine the ERα levels in the MCF-7 clones and how oestrogen affects the expression of the receptor.

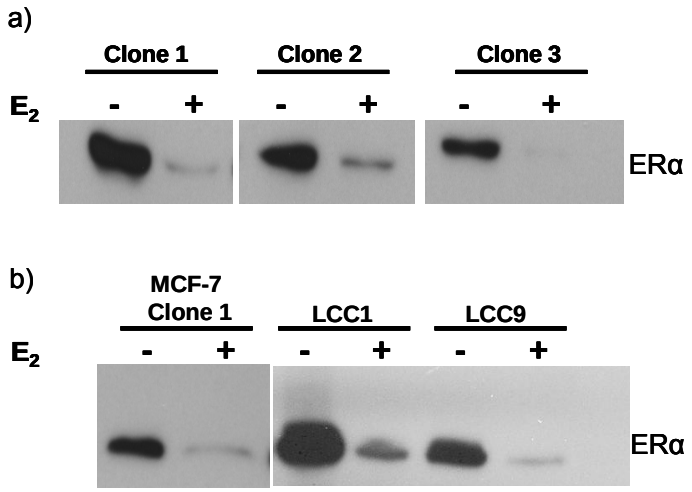


Figure 3.12 Western Blot analysis of ERα levels & oestrogen regulation of ERα in MCF-7 clones. (a) Oestrogen downregulates ERα in the three clones **(b)** MCF-7 clones have lower basal ER levels than LCC1 cells as previously described (Kuske et al, 2006).

ERα is expressed in the three clones and the protein is downregulated by oestrogen (Figure 3.12a) as described in previous reports (Kuske et al, 2006). MCF-7 clone 1 was used to determine how the ERα expression in the clones relates to the expression in LCC1 and LCC9. As shown in Kuske et al (2006), ERα expression is higher in LCC1 cells in comparison to clone 1. Additionally, ERα levels are similar in MCF-7 clone 1 and in LCC9 cells (Figure 3.12b).

3.2.3.2 Transcriptional Analysis

Transcriptional regulation of pS2 and ERα were also investigated. As an oestrogen regulated gene, pS2 mRNA expression is markedly upregulated in the presence of oestrogen. In the three clones, pS2 mRNA expression is responsive to oestrogen, doubling after 24h and quadrupling after 48h (Figure 3.13a). However, the response to oestrogen after 48h oestrogen treatment is not as pronounced in clone 3 as in clones 1 and 2.

Statistically, changes in pS2 expression are most significant and consistent in clone 2. Oestrogen activates transcription of genes which in turn promote cell growth such as proteins involved in cell cycle regulation, cell adhesion and DNA replication.

In contrast to pS2's transcriptional regulation, oestrogen downregulates ERα transcription. Expression of ERα mRNA was reduced in all three MCF-7 clones following oestrogen treatment (Figure 3.13b). The more consistent and significant results are once again observed in clone 2.

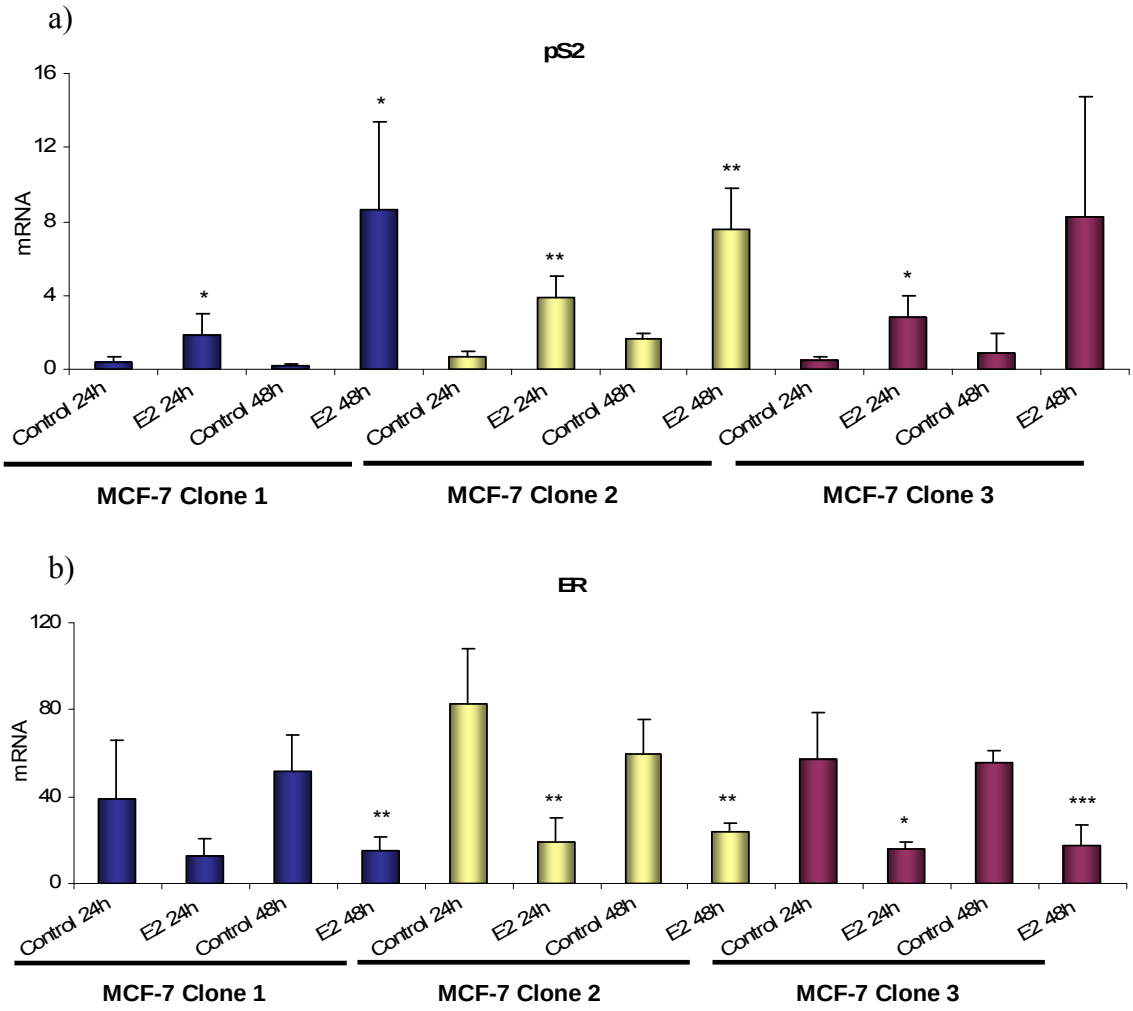


Figure 3.13 Regulation of oestrogen responsive pS2 (a) and ERα (b) genes in MCF-7 clones. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars=SD. Statistical analyses between each control vs E_2 (Student's paired t test *P<0.05; **P<0.01; ***P<0.001).

3.3 Signalling Pathways: Basal Characterisation

The three isolated MCF-7 clones behaved as expected according to literature on MCF-7 breast cancer cell line. They are oestrogen dependent, ERα positive and the regulation of protein/mRNA can be mediated by oestrogen. Nevertheless, there are a few subtle differences between the clones. The growth of clone 3, for example, appeared to be slower than the other two clones. In addition, oestrogen-mediated transcriptional regulation is only consistently significant in MCF-7 clone 2.

In order to conduct further experiments one MCF-7 clone had was picked for comparison with LCC1 and LCC9 cells. Clone 2 was chosen and from this point onwards will be referred to as MCF-7 only.

3.3.1 Epidermal Growth Factor Receptor Family

Overexpression of EGF receptor family members is often implicated in breast cancer, particularly HER2 so it was important to establish the role of this family in this endocrine resistant model.

Protein expression of EGFR is undetectable in MCF-7, LCC1 and LCC9 cells by standard western blotting although at the transcriptional level EGFR mRNA is significantly lower in oestrogen independent LCC1 and resistant LCC9 cells (Figure 3.15). HER2 protein expression levels are certainly not elevated in LCC1 and LCC9 cells, in fact they appear to be slightly lower when compared to the MCF-7 cell line (Figure 3.14). This appears to be a result of reduce transcription however the values are not statistically significant in LCC9 cells (Figure 3.15). Interestingly, HER3 mRNA levels are reduced in LCC1 and LCC9 in comparison to the parental MCF-7 cell line (Figure 3.14). However, HER3 protein levels are only slightly lower in LCC9 (Figure 3.15). Of all the EGF receptor family members, HER4 is the only one whose protein is overexpressed in LCC1 and LCC9 at the basal and phosphorylated level (Figure 3.14 & 3.15). This is not the case at the transcriptional level which indicates that elevated protein expression is a result of altered post-transcriptional regulation.

These results suggest that endocrine resistance in this three stage model is not being driven as a result of EGFR, HER2 or HER3 overexpression. HER4 expression, on the other hand, is elevated in LCC1 and LCC9 cells indicating that this receptor may be involved in endocrine resistance.

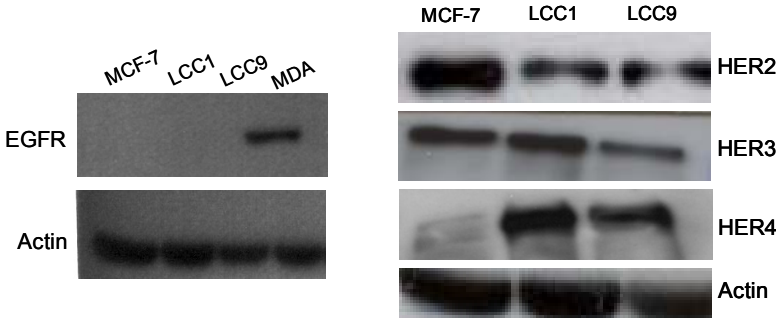


Figure 3.14 Expression of EGF Receptor Family. Western blot analysis of EGFR, HER2, HER3 and HER4 in MCF-7, LCC1 and LCC9. The MDA-MB 231 cell line (ER-negative) was used as a negative control as it is known to overexpress EGFR

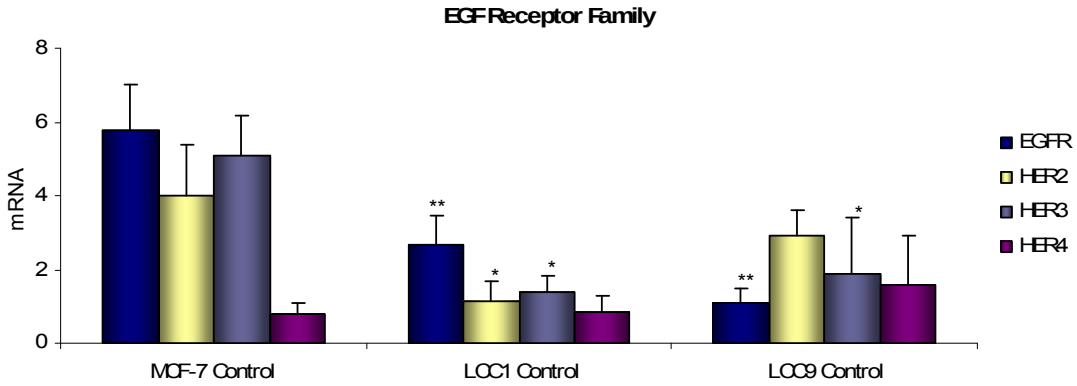


Figure 3.15 Transcription of EGF Receptor Family. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars=SD. Statistical analyses between MCF-7 vs LCC1/LCC9 for each receptor (ANOVA test *P<0.05; **P<0.01; ***P<0.001).

3.3.2 MEK/ERK Pathway

The MEK/ERK pathway is important in regulating cell proliferation and transcriptional control (Kurokawa *et al*, 2003). Furthermore, this pathway has been shown to be able to phosphorylate ERα at the ser118 residue and therefore is often implicated in resistance to endocrine resistance in breast cancer (Kato *et al*, 1995).

Activation of MEK and its downstream target ERK are similar in all three cell lines (Figure 3.16). This result indicates that this particular pathway is not important in conferring resistance in LCC1 and LCC9.

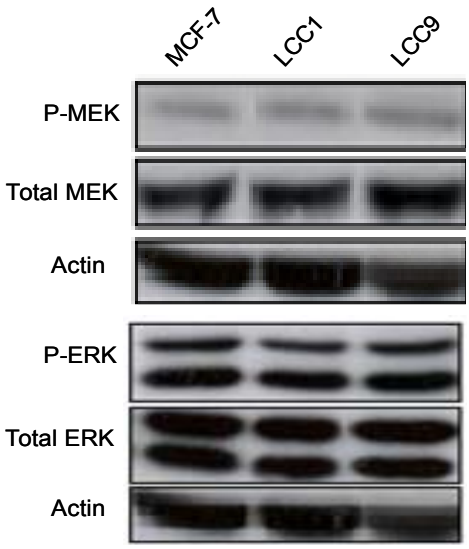


Figure 3.16 MEK/ERK Pathway. Western blot analysis of MEK and ERK (basal & phosphorylated levels).

3.3.3 PI3K/Akt Pathway

The PI3K/Akt pathway mediates cellular processes such as cell cycle entry, migration and cell survival (Shtilbans *et al*, 2008). Similarly to the MEK/ERK pathway, Akt has the ability to phosphorylate Ser167 of ERα in the absence of oestrogen. Phosphorylation of Akt at Ser473 is elevated in LCC1 and LCC9 whilst the basal levels remain similar in the three cell lines (Figure 3.17).

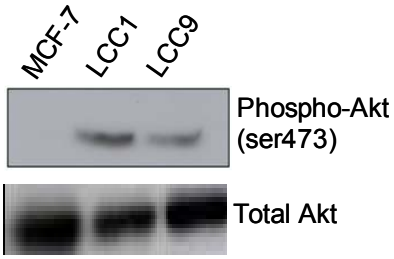


Figure 3.17 Akt Pathway. Western blot analysis of Akt levels & phosphorylation (Ser473). Results shown representative of three separate experiments

It is likely that increased phospho-Akt (Ser473) is involved in oestrogen independence and endocrine resistance. LCC1 and LCC9 cells may have the ability to activate ERα bypassing the need for oestrogen. This pathway and the role in this cell model are further discussed in Chapter 6.

3.4 ERα: its role in this model of endocrine resistance

3.4.1 ERα Basal Levels & Turnover

Oestrogen regulation of ERα is invariably at the forefront of endocrine resistant studies. Loss of ERα expression is one of the major mechanisms involved in conferring resistance to endocrine therapies. LCC1 and LCC9 cells remain ERα positive (Figure 3.18) and the receptor expression is downregulated by oestrogen treatment as previously shown (Kuske et al, 2006). Interestingly, the expression of ERα is higher in LCC1 cells than in the parental MCF-7 cell line.

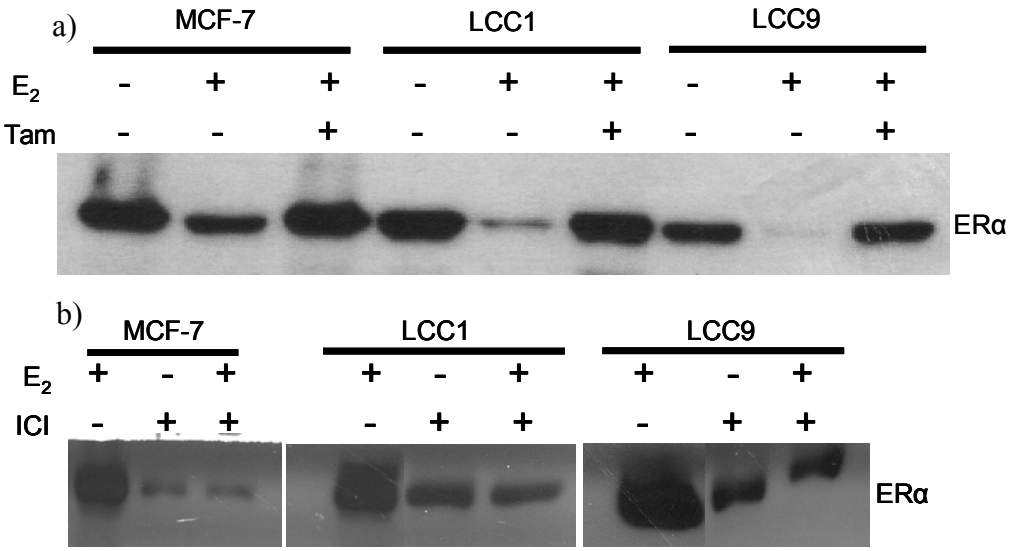


Figure 3.18 Western Blot Analysis of ERα levels & oestrogen regulation. Effects of Tamoxifen **(a)** & ICI 182,780 **(b)** on ERα regulation & protein expression in MCF-7, LCC1 and LCC9. Results representative of three independent experiments.

ERα expression is reduced in the presence of ICI (Figure 3.18b) but not tamoxifen (Figure 3.18a) in the MCF-7 cell line. This is also the case in the oestrogen independent LCC1 and fully resistant LCC9 cells. These results suggest that the regulation of ERα expression following treatment with anti-estrogens is not lost in this cell line model. Thus, LCC1 and LCC9 cell lines appear to retain ERα expression and respond to oestrogen and anti-oestrogens in a manner similar to that observed in parental MCF-7 cells.

3.4.2 ERα Basal Phosphorylation

As shown above, basal ERα expression and regulation appears to be maintained in LCC1 and LCC9 cell lines in comparison to the parental MCF-7 cells. However, it is also important to determine whether ERα activation is altered in this endocrine resistance model. As can be observed in Figure 3.19, Ser118 phosphorylation is certainly not elevated in LCC1 and LCC9 cells.

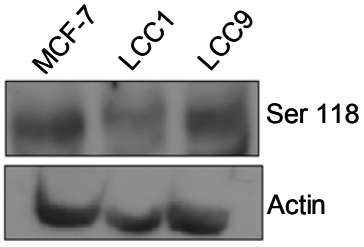


Figure 3.19 Western Blot analysis of ERα phosphorylation. Results shown representative of three independent experiments.

Consequently, it appears that phosphorylation at this residue is not directly associated with resistance in this cell line model. Phosphorylation at the Ser167 was also measured and the results are shown in Chapter 6 as they directly relate to Akt function.

3.5 Regulation of Cellular Mechanisms & Endocrine Resistance

In order to better understand endocrine resistance it was important to determine not only which pathways may be important in mediating resistance but also which cellular processes might be altered. Cell cycle, apoptosis, migration and invasion were all analysed.

3.5.1 Cell Cycle Analysis

MCF-7 growth in the absence of oestrogen is minimal and the cells are said to remain static. This is the result of a block at the G_0-G_1 cell cycle phase (Figure 3.20a) therefore all the treatments were administered in the presence of oestrogen. Herceptin and 2C4 did not affect cell cycle in MCF-7 cells. ICI 182,780 treatment, on the other hand, induces a significant increase in the percentage of cells at G_0-G_1 and significantly reduces the number of S phase cells.

Similarly to MCF-7 cells, LCC1's cell cycle is only affected by ICI 182,780 treatment (Figure 3.20b) in the same way as previously observed in the parental cell line. All other drug treatments do not induce a change in the cell cycle profile in comparison to control.

Cell cycle distribution in LCC9 cells was unaffected by any of the drug treatments (Figure 3.20c).

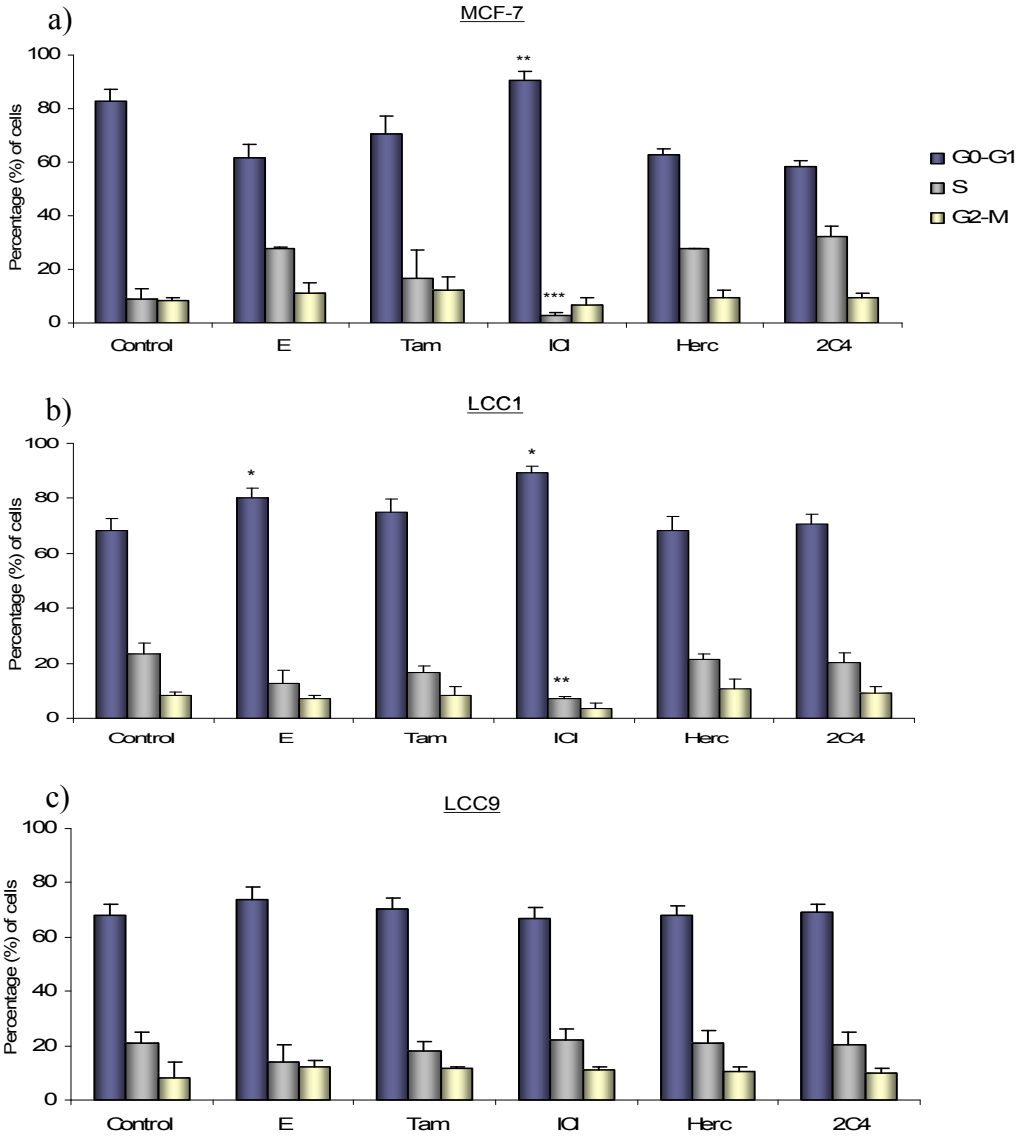


Figure 3.20 Cell Cycle Analysis. Effects of drug treatments on cell cycle of MCF-7 (a), LCC1 (b) and LCC9 (c). Each column presents means of three independent experiments. Error Bars=SD. Statistical analyses between E_2 vs treatment for each phase in MCF-7 cells and between control vs treatment for each phase in LCC1 & LCC9 cells (ANOVA test **P<0.01; ***P<0.001).

3.5.2 Apoptosis

Following observations showing that phosphorylation of the anti-apoptotic Akt is elevated in LCC1 and LCC9 cells it was crucial to establish whether apoptotic control was affected in endocrine resistance. Poly(ADP-Ribose) polymerase (PARP) cleavage was used as a marker or cells undergoing apoptosis. PARP (116 kDa) is involved in DNA repair and is important in mediating responses to damage. In apoptotic cells, PARP is targeted for proteolytic cleavage generating the 89kDa sized fragment.

In LCC1 cells, apoptosis as indicated by PARP cleavage is elevated following treatment with tamoxifen, herceptin and 2C4 (Figure 3.21). Furthermore, ICI treatment also induces apoptosis but the effects are not as pronounced as with the other inhibitors.

Apoptosis levels in LCC9 cells do not appear to be particularly affected by any of the drug treatments (Figure 3.21). There are a certain number of cells undergoing apoptosis in LCC9 cells as indicated by the presence of the 89kDa fragment, however the ratio of cleaved to uncleaved PARP remains relatively unchanged regardless of treatment.

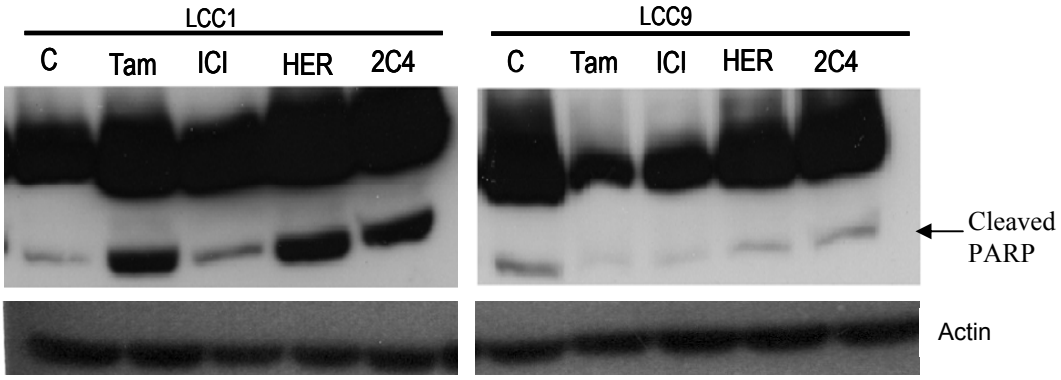


Figure 3.21 Apoptosis levels in LCC1 and LCC9 cells. Western Blot analysis for PARP cleavage, a well known marker of apoptosis.

3.5.3 Migration and Invasion

Migration and invasion were also measured in these three cell lines. A Boyden Chamber system was used to analyse cell migration in MCF-7, LCC1 and LCC9 cells in the presence of three different extracellular matrix proteins: collagen I, fibronectin and vitronectin. Cell migration is mediated by a number of different factors which in turn modulate ECM proteins.

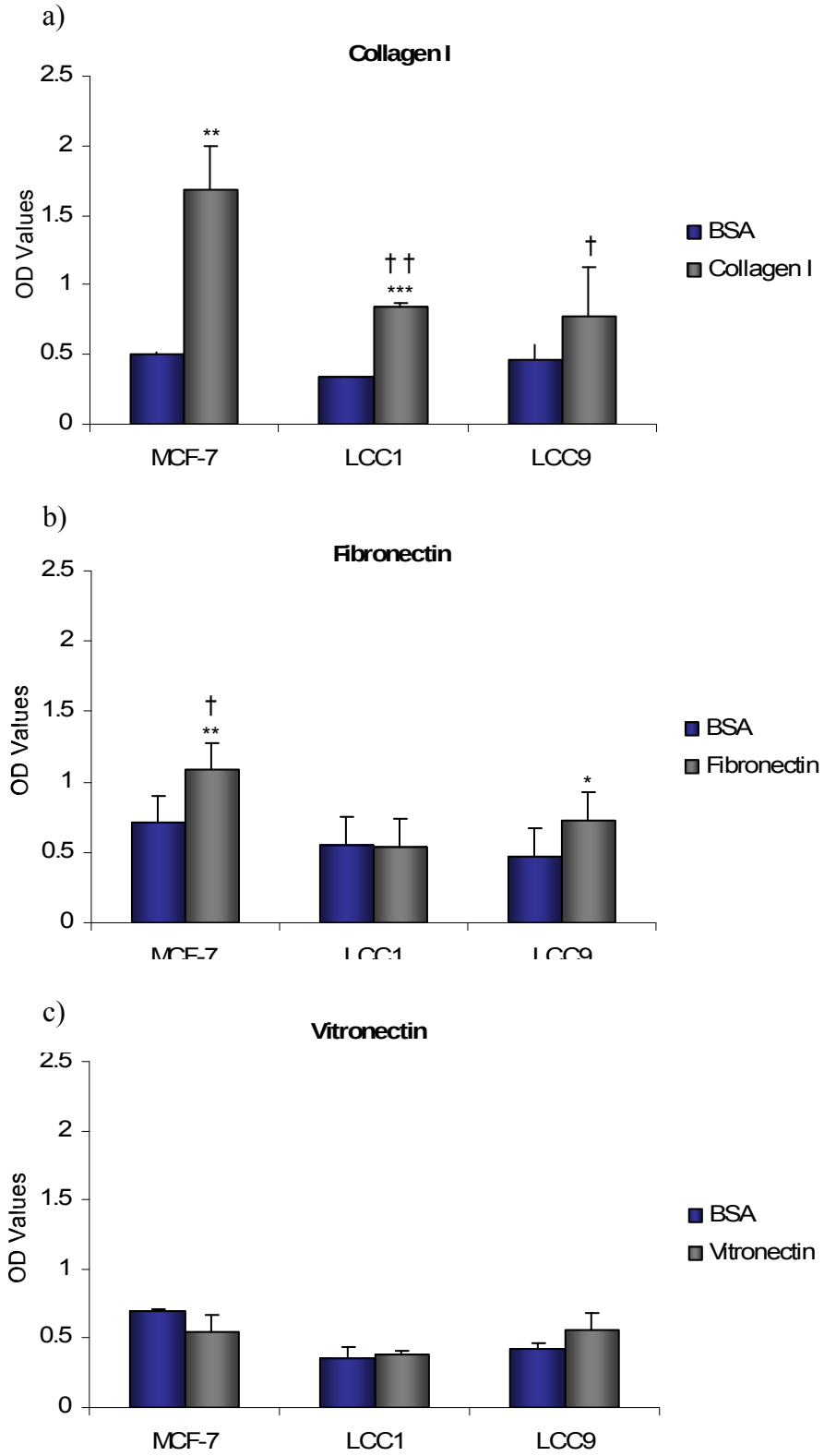


Figure 3.22 Migration levels in MCF-7, LCC1 & LCC9. The number of migrating cells was measured using three matrix proteins: Collagen I (a), Fibronectin (b) & Vitronectin (c). Each column presents means of four independent experiments. Error Bars=SD. Statistical analyses between BSA control vs sample for each cell line (Student's paired t test *P<0.05; **P<0.01; ***P<0.001) and MCF-7 vs LCC1/LCC9 (Student's unpaired t test †P<0.05; †P<0.01).

Collagen I and fibronectin induced migration at least in one of the cell lines whilst Vitronectin did not appear to do so (Figure 3.22). The number of MCF-7 migratory cells is significantly elevated in the presence of collagen I (Figure 3.22a) and fibronectin (Figure 3.22b) in comparison to BSA control. Migration in LCC1 cells was only significantly increased in collagen I treated chambers whilst migration in LCC9 cells was significantly stimulated only in the presence of fibronectin. Furthermore, comparison between the numbers of migratory MCF-7 cells versus LCC1/LCC9 cells revealed that the parental cells appear to be the most migratory. However this is only observed in Collagen I treated chambers (Figure 3.22a).

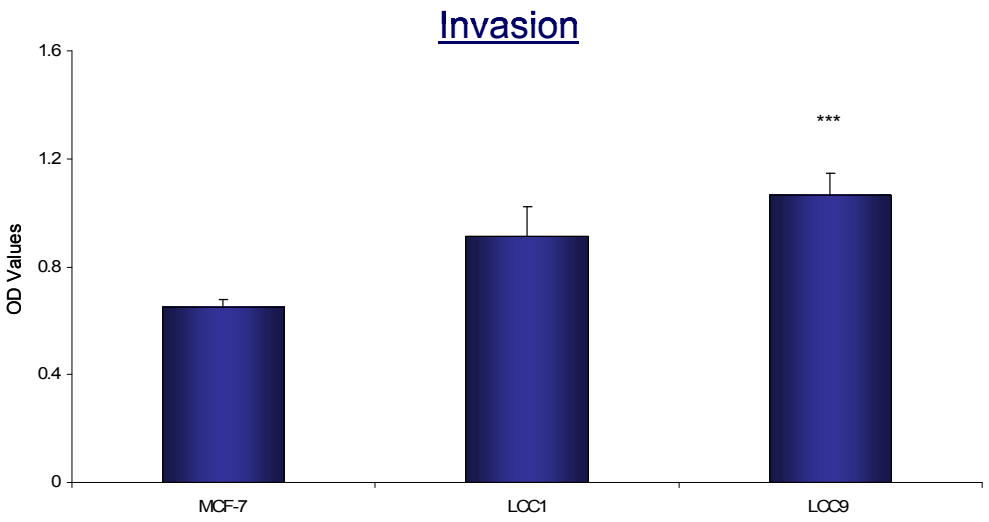


Figure 3.23 Invasion levels in MCF-7, LCC1 & LCC9. Each column presents means of four independent experiments. Error Bars=SD. Statistical analyses between MCF-7 vs LCC1/LCC9 (Student's unpaired t test ***P<0.001).

Invasion was the last of the cellular processes to be examined. The assay was designed to measure the ability of cells to move through a thin layer of extracellular matrix. Invasion ability is progressively increased in LCC1 and LCC9 cell lines respectively in comparison to MCF-7 cells. However, invasion ability is only significant elevated in LCC9 cells (Figure 3.23).

3.6 Discussion

Various cellular processes were fully characterised in the MCF, LCC1 and LCC9 cell lines and these included growth, apoptosis, cell cycle, migration and invasion. Some of these

mechanisms have previously been studied and the results presented here are consistent with published data (Brunner *et al*, 1993; Brunner *et al*, 1997).

LCC1 and LCC9 cells were derived from MCF-7 cell lines hence these cells are considered to be the parental cell line and may be directly compared. As shown here and by previous studies (Power *et al*, 2003), MCF-7 cells are effectively static in the absence of oestrogenic stimulus and require this hormone in order to grow. They are also sensitive to the stimulatory effects of growth factors such as TGFα in agreement with published data (Keshamouni *et al,* 2002; Larsen *et al,* 1999). As reported by Kallio *et al* (2005) and Seeger *et al* (2003), tamoxifen inhibited oestrogen-driven MCF-7 cellular proliferation but had little effect when administered alone.

MCF-7 cells were grown in ovariectomized mice in order to select for oestrogen independent growth *in vivo*. The LCC1 cell line was isolated from tumours growing in these mice and, as shown in this chapter, is characterized as having acquired oestrogen independent growth. This is supported by previous studies by Brunner *et al* (1993), Clarke *et al* (1993) and Arteaga *et al* (1999). Nevertheless, LCC1 cellular proliferation is still oestrogen-sensitive. Unlike the parental MCF-7 cell line, growth of LCC1 cells is not stimulated by the addition of TGFα. However, these cells resemble MCF-7 cells since they are sensitive to anti-oestrogens tamoxifen and ICI 182,780.

In contrast to LCC1 cells, the LCC9 cell line was found to be completely insensitive to anti-oestrogens and growth factor/oestrogen stimulation which has been previously shown by a number of groups (Brunner *et al*, 1997; Clarke *et al*, 1987).

Morphologically, MCF-7 cells look quite different from its derivative cell lines. Under control conditions without oestrogenic stimulation, MCF-7 cells have a flat “cobblestone” appearance and grow as adherent epithelial-like cells. The cell appearance is only altered in the presence of oestrogen which appears to promote cell-cell adhesion rather than cell-matrix adhesion observed in the control as previously observed by Srivastava *et al* (2004). The study by Srivastava *et al* (2004) also suggested that tamoxifen treatment did not phenotypically alter MCF-7 cells whilst Stewart *et al* (1992) reported no morphologic alterations following TGFα treatment.

The morphology of LCC1 and LCC9 cells was not as structured and organised as the parental MCF-7 cell line. Interestingly, MCF-7 cells treated with oestrogen adopt morphology not dissimilar to that of LCC1 and LCC9 cells. This may indicate that the

MCF-7 derivatives preferentially form cell-cell adhesion thereby giving rise to a more "clumpy" appearance.

MCF-7 cells have become the most commonly used model for ER-positive breast cancer. The MCF-7 cell line is now distributed in laboratories throughout the world so it is not surprising that clonal variations have been reported since MCF-7 cells were first established in 1973 (Nugoli *et al*, 2003). Such clones mainly differ in their ability to respond to oestrogen and in their tumour formation ability (Seibert *et al*, 1983; Whang-Peng *et al*, 1983; Butler *et al*, 1986). This was further demonstrated by Resnicoff *et al* (1987) as they have shown that MCF-7 fractionation gave rise to six different subpopulations. Most importantly, one of these cell lines bore the ability to generate all other 6 populations suggesting MCF-7 cells contain a fraction of stem cells which may generate clonal variability (Resnicoff *et al*, 1987; Nugoli *et al*, 2003).
The MCF-7 cell line first used in this study was found to comprise of (at least) two cellular populations as observed by cell cycle analysis. As described above this is not an uncommon event however it was important to try to eliminate clonal variation is these cells to aid the interpretation of future results, particularly cell cycle studies. Three clones were isolated from the MCF-7 cell line and all three were fully characterised in term of phenotype, oestrogen response, ERα protein expression and gene expression. One of these clones was chosen to perform all future experiments as it behaved as MCF-7 cells, in agreement with previously published reports.

The expression of EGF receptors and downstream signalling pathways in MCF-7, LCC1 and LCC9 cells was fully investigated in order to establish whether these mechanisms are important in conferring oestrogen in independence and endocrine resistance.
EGFR protein expression was undetected in all three cell lines however, EGFR transcription was significantly lower in oestrogen independent LCC1 and resistant LCC9 cells. EGFR overexpression in breast cancer was a strong indicator for recurrence and is associated with reduced disease-free survival (Normanno *et al*, 2005). Additionally, EGFR expression is generally increased in ER-negative breast cancers (Normanno *et al*, 1994; Normanno *et al*, 2005) whilst EGFR signalling can promote tamoxifen resistance (Nicholson *et* al, 2004; Dowsett *et al*, 2001; Ellis *et al*, 2001). Similarly to EGFR expression, HER2 is not elevated in LCC1 and LCC9 suggesting overexpression of these receptors which is often implicated in endocrine resistant in breast cancer. The HER2

receptor became a major therapeutic target in breast cancer treatment following reports that this receptor is overexpressed in 15 to 30% of breast tumours (Ellis *et al*, 2004). However, the results presented here suggest the development of endocrine resistance in the cell line model used in this study is not dependent on the overexpression of the EGFR and HER2 receptors unlike many of the other cell line models.

HER3 overexpression has been observed in an average of 25% of invasive breast carcinomas (Karamouzis *et al*, 2007). HER3 expression is linked to HER2 positivity (Gasparini *et al*, 1994) and co-expression of the two receptors has been shown to significantly promote mammary tumourigenesis (Holbro *et al*, 2003) which is not surprising considering that the HER2/HER3 heterodimer has the most potent mitogenic ability of all the heterodimerisation possibilities (Citri *et al*, 2003). HER3 protein expression is relatively unchanged in LCC1 and LCC9 cells despite transcription being significantly reduced in comparison to parental MCF-7 cell line. Various studies have shown that HER3 plays a role in resistance to treatment, particularly to EGFR tyrosine kinase inhibitors and HER2-targeted therapies were it may compensate and help cells escape the inhibitory effects of these drugs (Kong *et al*, 2008; Normanno *et al*, 2009; Koutras *et al*, 2009). Of the four members of the EGF receptor family, HER4 was the only member overexpressed in LCC1 and LCC9 cell lines. This is surprising considering HER4 activation is linked to antiproliferative and pro-apoptotic activity. Furthermore, HER4 expression correlates with favourable prognostic factors such as ER/PR positivity, low histopathological grade (Witton *et al*, 2003) and increased overall survival in breast cancer (Koutras *et al*, 2008). However, recent studies reported HER4 mRNA levels was associated with poor outcome and reduced relapse-free survival in a subset of breast cancer patients (Bieche *et al*, 2003). HER4 receptor cleavage generates a soluble HER4 intracellular domain (4ICD) and the localization of this domain appears to determine HER function thereby explaining the aforementioned contrasting results regarding HER4 as a prognosis indicator (Ni *et al*, 2001; Junttila *et al*, 2005).

These results together suggest that direct deregulation of EGF, HER2 and HER3 expression is not directly linked to endocrine resistance. However, HER4 expression is markedly elevated in oestrogen independent LCC1 and resistant LCC9 cells. Expression of the soluble intracellular domain and its localization was not determined in these cell lines however in this model HER4 appears to be associated with increased tumour formation ability, oestrogen independent growth and resistance to endocrine therapies.

Epidermal growth factor receptors mediate a number of cellular processes by activating a variety of downstream signalling pathways such as the ERK and Akt pathways. Elevated MEK/ERK activation is observed in oestrogen unresponsive and resistant MCF-7 cells (Santen *et al*, 2005; Normanno *et al*, 2006). Furthermore, the MEK/ERK pathway has the ability to phosphorylate and consequently activate the ERα receptor in the absence of oestrogenic stimulation (Kato *et al*, 1995). MEK and ERK levels are unchanged in LCC1 and LCC9 cells, as previously reported (Kuske *et al*, 2006). Akt activation was also studied in this cell line model and Akt phosphorylation (Ser473) was shown to be increased in LCC1 and LCC9 cells suggesting this pathway may be important in conferring resistance. The Akt pathway is discussed in detail in Chapter 6.

As previously reported (Kuske *et al*, 2006), LCC1 and LCC9 cells retain functional ERα expression since oestrogen still reduces ERα protein levels. It has been proposed this is due to proteasomal degradation, a process known to limit oestrogen signalling (Nawaz *et al*, 1999). In addition, basal ERα expression is increased in LCC1 cells but less so in LCC9 cells in comparison to the parental MCF-7 cell line as previously shown (Kuske *et al*, 2006). Tamoxifen did not reduce ERα expression in any of the three cell lines whilst ICI 182,780 reduced ERα levels in MCF-7, oestrogen independent LCC1 and resistant LCC9 cell lines. These results suggest that oestrogen is still binding and activating the ERα in resistant cell lines as demonstrated by ERα protein turnover and that expression of this receptor is still regulated by anti-oestrogens in a manner similar to that of the parental cell line.

Phosphorylation of ERα at the Ser118 was not elevated in resistant LCC9 cells and appears to be slightly reduced in LCC1. This indicates that endocrine resistance in this cell line model is not mediated by increased activation at the serine residue. The other main ERα phosphorylation site (Ser167) was also studied and the results are discussed in Chapter 6.

Oestrogen and its receptor have also been shown to regulate cell cycle machinery (Planas-Silva *et al*, 1997) therefore it was important to determine whether cell cycle is altered in endocrine resistance. Under control conditions in the absence of oestrogen, MCF-7 cells arrest in G_0/G_1 phase of the cell cycle as previously reported (Lukyanova *et al*, 2009). Oestrogen stimulation significantly increases the percentage of cells in S-phase of cell cycle which was attenuated to some extent in the presence of tamoxifen and fully

following ICI 182,780 treatment. These results are in agreement with previously published results (Prall *et al*, 1997; Abdelrahim *et al*, 2002; Hodges *et* al, 2003; Riggins *et al*, 2005).
In contrast to the parental cell line, the percentage of LCC1 and LCC9 cells at the S-phase was markedly increased in relation to MCF-7 cells. This supports the cellular proliferation results which show increased growth rates of LCC1 and LCC9 cells in the absence of ligand stimulation. Furthermore, oestrogen stimulation did not induce cell cycle progression as observed in parental MCF-7 cells. In fact, the percentage of LCC1 cells in S-phase is slightly reduced post-treatment presumably due to the superfluous amount of ligand interfering with the cell cycle machinery. LCC1 and LCC9 cells also differ in their response to ICI 182,780 treatment since this anti-oestrogen induces G_0/G_1 arrest in LCC1 but not in resistant LCC9 cells as previously reported (Riggins *et al*, 2005).
Herceptin and 2C4 had little effect in the cell cycle of all three cell lines. Herceptin is thought to inhibit cell cycle progression by up-regulating expression of the $p27^{KIP1}$ family of cyclin inhibitors thereby promoting arrest at the G_0/G_1 phase. *In vivo* studies, however have observed no herceptin-mediated cell cycle arrest in xenografts suggesting herceptin promotes transient cell cycle arrest (Warburton *et* al, 2004; Lane *et al*, 2000; Sliwkowski *et al*, 1999). The effects of herceptin on cell cycle were expected to be limited since MCF-7, LCC1 and LCC9 cells do not overexpress HER2. However, 2C4 which does not require HER2 overexpression, also has little effect suggesting that blocking HER2 heterodimer formation does not play a part in cell cycle regulation in these cell lines.
Oestrogen has been shown to promote G_1 progression by activating cyclin D1 which in turn activates Cdk4 and Cdk2 which regulate G_1-S transition (Prall *et al*, 1997). Murray *et al* (2005) have reported that cyclin D1 expression is elevated in mammary tumours versus normal mammary tissue whilst Hodges *et al* (2003) observed that this cell cycle regulator is often upregulated in tamoxifen resistant breast cancer cells. Hence, cyclin D1 may be important in promoting cell cycle transition in oestrogen-independent LCC1 and resistant LCC9 cell lines in the absence of ligand stimulation.

The apoptotic response of these cells to a variety of treatments was also established in LCC1 and LCC9 cells. Apoptosis levels were determined by measuring the levels of PARP cleavage. The PARP enzyme is implicated in DNA damage and repair mechanisms. During apoptosis, PARP is cleaved by caspase- 3 thereby generating an 85kDa fragment from the native 116 kDa sized protein (Oliver *et al*, 1998). Most drug treatments induced

apoptosis in LCC1 cells, particularly tamoxifen, herceptin and 2C4. In resistant LCC9 cells, on the other hand, apoptosis is markedly reduced. Reduced apoptotic drive in LCC9 has been linked to the expression of the transcription factor interferon regulator factor-1 (IRF-1). This tumour suppressor is downregulated in LCC9 cells and stable transfection of dominant-negative IRF-1 eliminated ICI 182,780-mediated apoptosis in MCF-7 cells (Bouker *et al*, 2004). Furthermore, the nuclear factor κB (NFκB) transcription factor family has also been implicated in the apoptotic control of this MCF-7-based cell line model. The expression of two members of this nuclear factor family, the p65 subunit and the upstream regulator IκB, is elevated in the resistant LCC9 cell line and inhibition of these proteins restored ICI 182,780 sensitivity in these cells (Riggins *et al*, 2005).

Finally, the cellular motility and invading ability of these cell lines was also investigated. Migration was analysed in the presence of three difference extracellular matrix proteins, collagen, fibronectin and vitronectin. Collagen was the most effective in promoting cellular migration in all three cell lines whilst vitronectin had little effect. However, in the presence of collagen and fibronectin, MCF-7 cells appear to be the most migratory of the three cell lines. This is supported by their phenotypes and growth characteristics since the parental cell line grows as a flat sheet of cells, whilst LCC1 and LCC9 cells tend to form "clumps". Nevertheless, previous studies have reported an association between increase migration and tamoxifen resistance (Castro *et al*, 2005; Hiscox *et al*, 2006).

Invasion, on the other hand, is slightly increased in oestrogen-independent LCC1 cells and significantly elevated in endocrine resistant LCC9 cells in contrast to the MCF-7 cell line. This is in agreement with previously published results which show tamoxifen resistant MCF-7 cells display a loss of cell-cell junctions and overexpress proteins involved in epithelial to mesenchymal transition an important step in progression and metastasis of breast cancer (Ree *et al*, 1998; Hiscox *et al*, 2006; Planas-Silva *et al*, 2005).

Chapter 4

EGF Receptor Family: Oestrogen Regulated Transcriptional Control and p160 Co-activators

A number of studies have previously shown that oestrogen potently inhibits HER2 transcription of ER-positive cell lines such as ZR-75-1, T47D and MCF-7 cells (Dati *et al*, 1990; Read *et al*, 1990; Russell *et al*, 1992). Furthermore, EGF treatment also suppresses HER2 protein expression but this is a result of post-translational alterations rather than at the transcriptional level (Antoniotti *et al*, 1994).

Expression of ERα is necessary and sufficient for oestrogen mediated *HER2* repression and this mechanism may be reversed by the addition of anti-oestrogens such as tamoxifen and ICI 182,780 (Russell *et al*, 1992; Taverna *et al*, 1994). The effect of oestrogen on HER2 expression is mediated by a 409 bp region found within the first intron of the *HER2* gene (Bates *et al*, 1997) and *in vitro* footprinting revealed that there are four transcription factor binding sites in this oestrogen-suppressible enhancer (Newman *et al*, 2000). The activator-protein 2 (AP-2) family of transcription factors consisting of five members (AP-2α; AP-2β; AP-2γ; AP-2δ; AP-2ε) is one of such transcription factors able to bind to *HER2*. Additionally, this protein family has been shown to drive HER2 overexpression often present in breast cancer cases (Eckert *et al*, 2005). The binding sites for AP-2 proteins are located within the transcription binding sites described by Newman *et al* (2000) suggesting the AP-2 family is required for oestrogenic suppression (Orso *et al*, 2004). ERα mutational analysis has shown that the DNA binding domain alone did not repress HER2 transcription. However, removal of the C-terminus containing the AF2 domain completely abrogated HER2 inhibition (Newman *et al*, 2000). This domain is important in activation following oestrogen stimulation and crucial in initiating the assembly of the transcriptional machinery. These results indicate that the regulation of HER2 repression by oestrogen essentially occurs off the DNA hence it is unlikely that the AP-2 proteins are required in this mechanism (Newman *et al*, 2000).

The region of ERα involved in the oestrogenic repression of HER2 is also important in recruiting p160 co-activators to the receptor in preparation for transcriptional activity suggesting that this cofactor family may be important in regulating this mechanism. This observation led to Newman *et al* (2000) proposing a model whereby activated ERα and *HER2* enhancer compete for binding of p160 co-activators leading to *HER2* inhibition. They found that SRC-1 transfection into ZR75-1 breast cancer cell lines relieved repression and restored HER2 enhancer activity. On the other hand, the two other co-activators TIF2 and AIB1 did not show significant effects (Newman *et al*, 2000).

According to these results, the p160 co-activator family is important in oestrogen mediated *HER2* transcriptional repression hence the role of these proteins in our cell line model was established.

4.1 Oestrogen Mediated Repression of HER Receptors

4.1.1 Oestrogen Downregulation of *HER2* is reduced in endocrine resistant cells

In MCF-7 and LCC1 cells, oestrogen treatment for 48h leads to a marked reduction in HER2 expression at the protein level which can be reversed by the addition of the anti-oestrogen ICI 182,780 (Figure 4.1a). Further analysis reveals that this is also observed at the transcriptional level (Figure 4.1b) since HER2 mRNA levels are significantly reduced in the presence of oestrogen (MCF-7 p-value 0.001; LCC1 p-value 0.007). In resistant LCC9 cells oestrogen mediated HER2 repression is far less prominent (Figure 4.1).

The effects of oestrogen are not only observed at the receptor level. Activation of the MEK/ERK pathway is also reduced after oestrogen treatment in MCF-7 cells and to a small extent in LCC1 cells. Phospho-ERK levels are particularly reduced in MCF-7 (Figure 4.2). Basal levels of MEK and ERK are not altered following oestrogenic stimulus. As previously observed for HER2 protein expression, the MEK/ERK pathway remains unchanged in LCC9 cells (Figure 4.2).

Oestrogen downregulation of MEK/ERK pathway is likely to be a result of the upstream HER2 repression. Membrane receptors such as HER2 are able to activate a variety of downstream pathways including the MEK/ERK pathway hence reducing HER2 protein expression is likely to affect phosphorylation of these downstream effectors. This is supported by the unchanged basal levels of MEK and ERK.

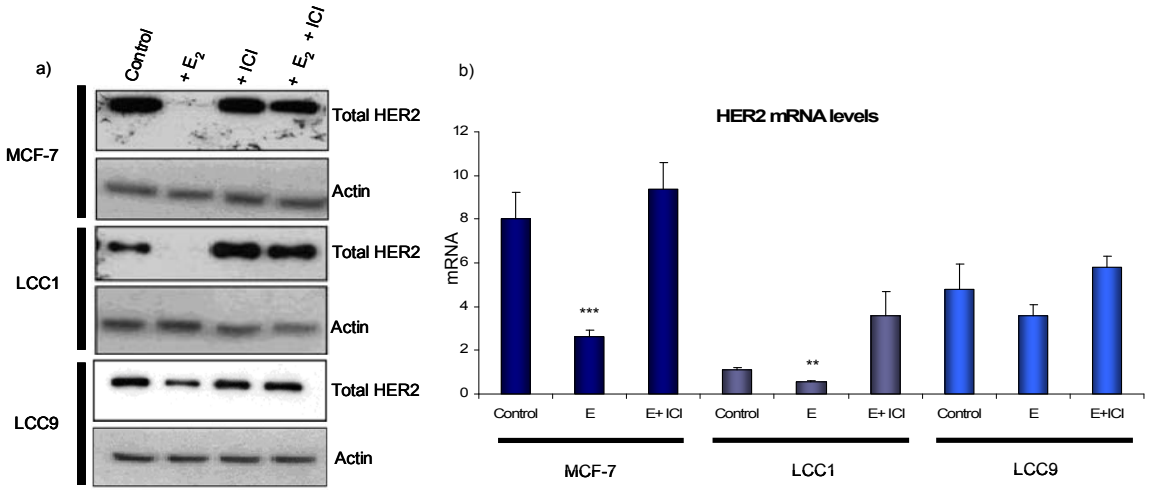


Figure 4.1 Oestrogen downregulates HER2 in MCF-7 and LCC1 but minimally in LCC9 cells. Cells were treated with E_2 (1nM) for 48h. Western Blotting was used to measure protein expression (a) (representative of three independent experiments) whilst mRNA levels were determined by quantitative RT-PCR (b). Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for oestrogen treatment vs matched control (Student's unpaired t-test **P<0.01; ***P<0.001).

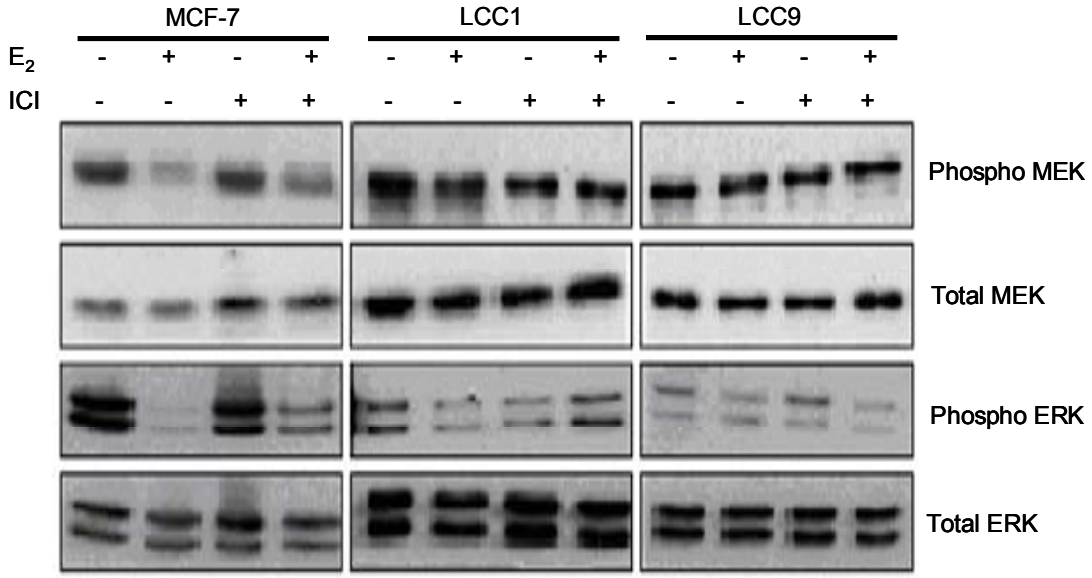


Figure 4.2 Effects of oestrogen on MEK/ERK pathway. Western blot analysis of pathways downstream from HER2. Results representative of three independent experiments.

4.1.2 Oestrogen Regulation of EGFR, HER3 & HER4

Following the observation that oestrogen was able to repress HER2 transcription it was important to determine whether this effect is restricted to HER2 or if other members of the EGF receptor family are also affected.

EGFR mRNA levels are unchanged in the presence of oestrogen in MCF-7, LCC1 and LCC9 cells (Figure 4.3). Protein expression could not be measured since EGFR protein is not detected by western blotting in these cells as previously shown in Chapter 3. Unlike EGFR, HER3 mRNA expression is also downregulated in the presence of oestrogen in MCF-7 and LCC1 cells, though the latter does not reach significance (Figure 4.4b). This is also observed at the protein level (Figure 4.4a). As previously observed for HER2, HER3 protein and mRNA levels in LCC9 cells remain unchanged.

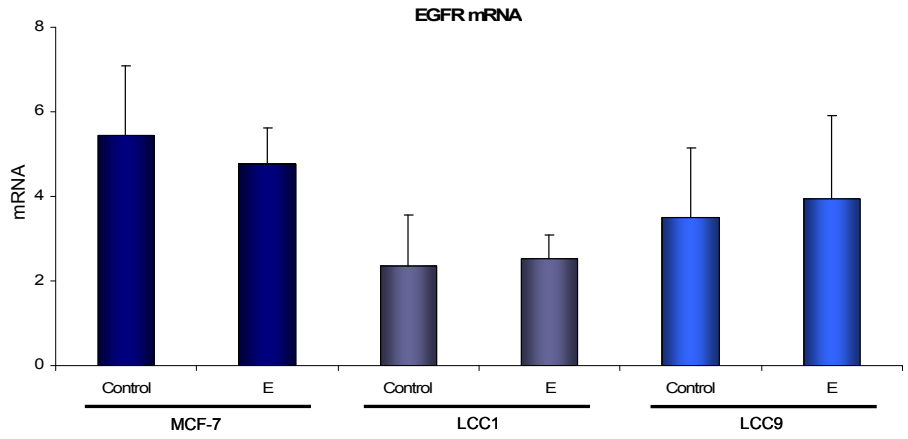


Figure 4.3 EGFR transcriptional regulation. Expression of EGFR mRNA by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD.

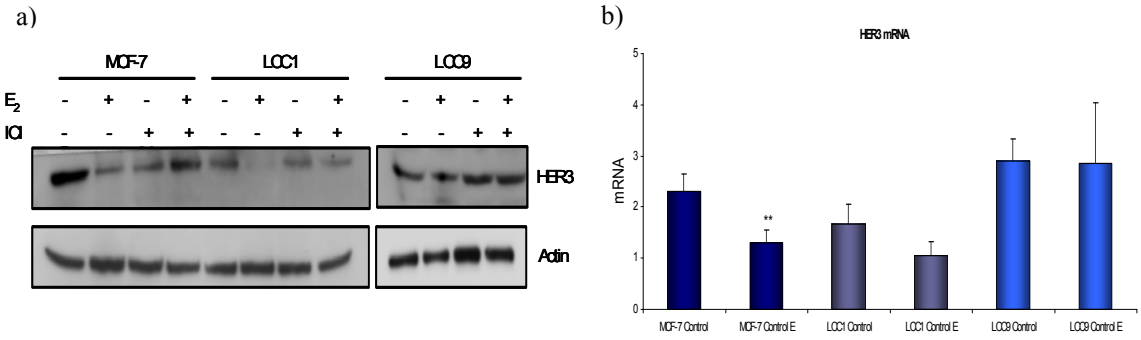


Figure 4.4 Oestrogen Regulation of HER3 Expression. (a) Protein expression was measured by western blotting. Results representative of three independent experiments (b) HER3 mRNA levels were determined by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for oestrogen treatment vs matched control (Student's unpaired t-test **P<0.01).

Similarly to HER2 and HER3, HER4 expression is downregulated by oestrogen in MCF-7 and LCC1 cells (Figure 4.5 & 4.6). MCF-7 cells express very low levels of HER4 hence protein was not detectable by western blotting. Nonetheless, oestrogen does reduce HER4 mRNA levels in MCF-7 suggesting that this may also be the case at the protein level. HER4 transcription in LCC9 cells is not responsive to oestrogen as previously observed (Figure 4.5 & 4.6).

These results show that not only does oestrogen mediate HER2 transcription it also affects the transcription of HER3 and HER4. Receptor expression is downregulated by oestrogen in endocrine sensitive MCF-7 and LCC1 cells but this is lost in the resistant LCC9 cells. This suggests that transcriptional regulation of these receptors is altered in endocrine resistant cells and this may play a role in conferring resistance.

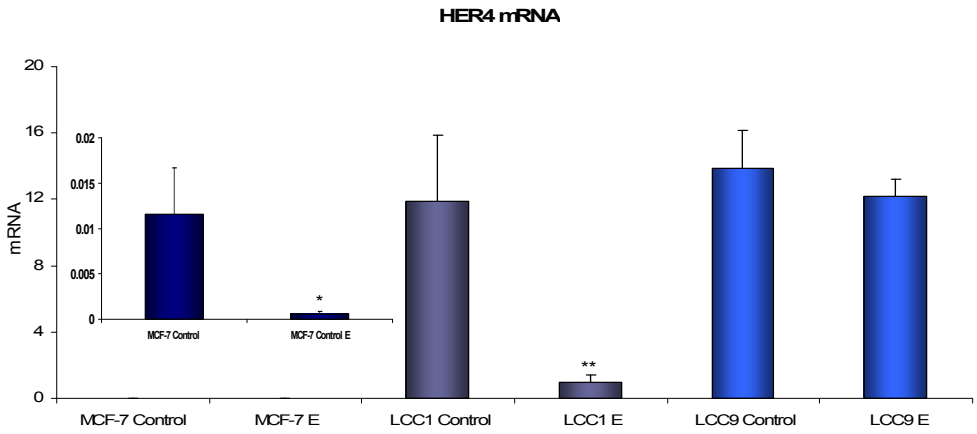


Figure 4.5 Oestrogen mediated transcriptional regulation of HER4. HER4 mRNA levels were determined by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for oestrogen treatment vs matched control (Student's unpaired t-test *P<0.05; **P<0.01).

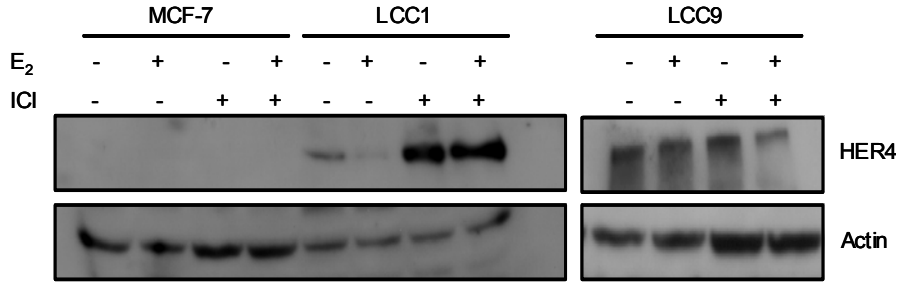


Figure 4.6 HER4 protein expression is downregulated by oestrogen. Protein expression was measured by western blotting. Results representative of three independent experiments.

4.2 HER2 Transcriptional Control: Role of ERα and SRC-1

Previous studies have demonstrated oestrogen mediated HER2 repression in several human breast cancer cell lines including MCF-7 which is reversed in the presence of tamoxifen and ICI 182,780 and the data presented here is in agreement with this (Dati *et al*, 1990; Read *et al*, 1990). HER2 transcriptional repression by oestrogen appears to be mediated by a region within the first intron of HER2 with the ability to act as an oestrogen-suppressible enhancer (Hurst *et al*, 1997). Reports have also shown that the AF2 domain of ERα is essential for HER2 transcriptional suppression (Newman *et al*, 2000). Furthermore, overexpression of SRC-1, a member of the p160 coactivator family, appears to relieve oestrogen repression in a dose dependent manner (Newman *et al*, 2000). Following these observations they proposed a model whereby ERα (through the AF2 domain) and HER2 enhancer compete for SRC-1. In the presence of oestrogen, SRC-1 preferentially binds to ERα consequently reducing HER2 transcription whilst in anti-oestrogenic conditions, SRC-1 is free to activate enhancer activity (Figure 4.7).

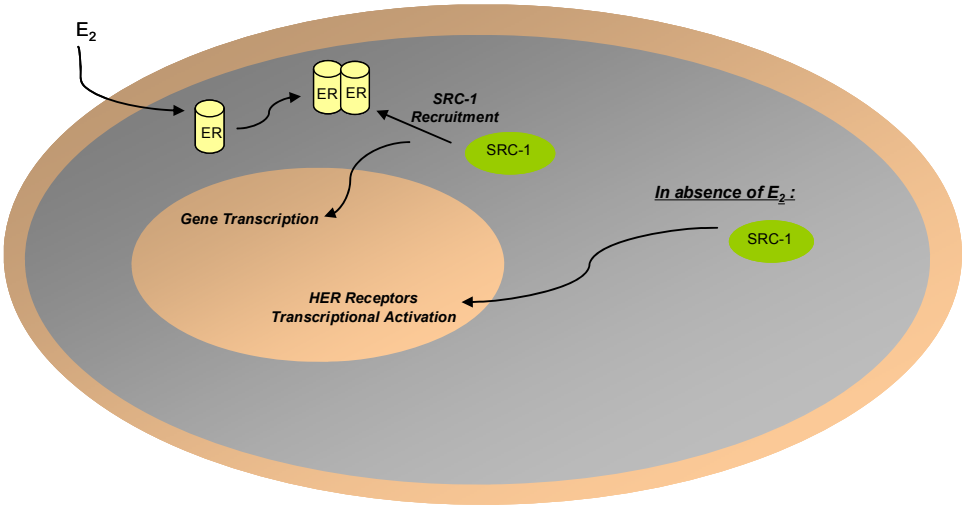


Figure 4.7 Proposed model for HER2 transcriptional regulation.

In our three step cell line model for endocrine resistance, the oestrogenic effects on transcription of HER2, HER3 and HER4 appear to differ between endocrine sensitive and endocrine resistant cell lines. According to previously published data, SRC-1 and ERα are important in the control of this mechanism hence the interactions between these two proteins were studied.

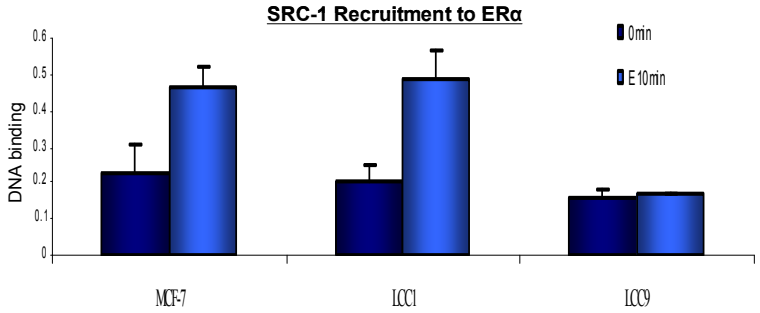


Figure 4.8 SRC-1 binding to ERα gene is observed in MCF-7 & LCC1 but not in LCC9 cells. Oestrogen induced SRC-1 recruitment to ERα promoter was determined by ChIP after 10 min treatment. Data was normalized to basal levels and presented as the mean ±SE for three independent experiments.

ChIP experiments performed in our laboratory by Dr Catherine Naughton have revealed that oestrogen triggered SRC-1 recruitment to the ERα gene is lost in LCC9 cells (Figure 4.8). In MCF-7 and LCC1 cells on the other hand, SRC-1 is recruited as part of the ERα-cofactor transcriptional complex after oestrogen treatment (Figure 4.7).

This data in combination with previously published results were used to outline a potential model for oestrogen mediated transcriptional repression of HER2 whereby in MCF-7 and LCC1 cells oestrogen treatment leads to ERα sequestering SRC-1 from HER2 enhancer consequently reducing transcription (Figure 4.9). In LCC9 cells SRC-1 does not appear to be recruited to ERα and is therefore able to bind to the promoter region of HER2. This model would explain why HER2 mRNA and protein levels are reduced in MCF-7 and LCC1 cells in oestrogenic conditions but not in LCC9 cells.

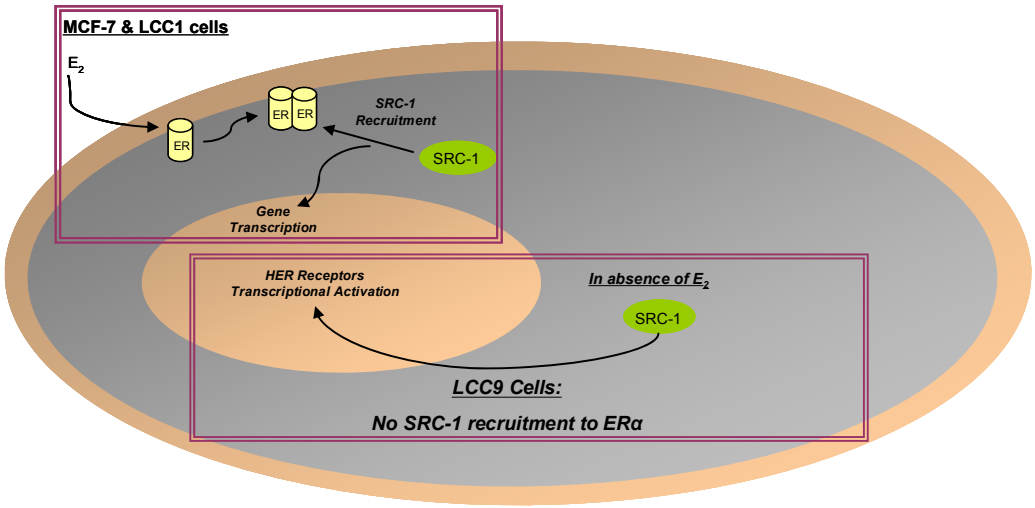


Figure 4.9 Proposed model for oestrogen-mediated HER2 transcriptional repression in endocrine resistance. In MCF-7 and LCC1 cells oestrogen induces SRC-1 recruitment to ERα and is therefore no longer available to bind to the HER2 promoter. In LCC9 cells, on the other hand, SRC-1 co-activator is not recruited to the oestrogen receptor hence it is free to activate HER2 transcription.

4.2.1 ERα RNAi Reverses Oestrogen-mediated HER2 Downregulation

According to the proposed model, HER2 repression is a result of competition between ERα and HER2 enhancer for SRC-1. If this is the case reducing ERα protein by siRNA would effectively restore HER2 protein expression in the presence of oestrogen. ERα RNAi restores HER2 protein levels to control levels in MCF-7 and LCC1 thereby reversing the effects of oestrogen. There is also a slight increase in HER2 protein expression in LCC9 cells (Figure 4.10). These results support the proposed model for HER2 transcriptional regulation however it is possible that ERα RNAi is having secondary effects such as displacing an ERα associated protein.

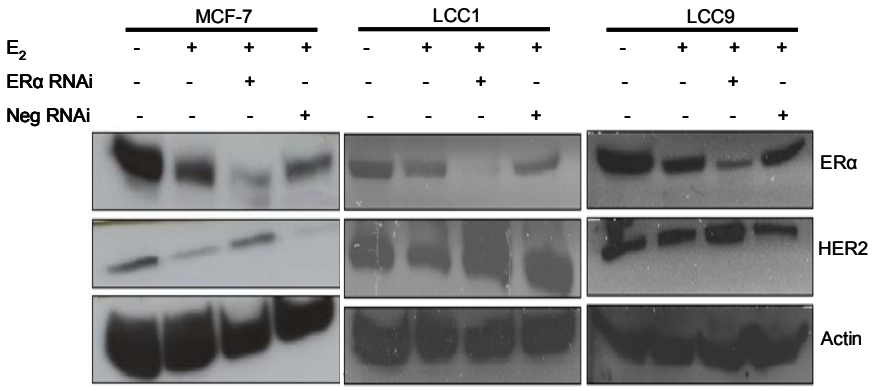


Figure 4.10 ERα RNAi reverses oestrogen repression of HER2 in MCF-7 cells. Western Blot analysis of HER2 protein expression following treatment with ERα siRNA. Results representative of four independent experiments.

4.3 Loss of SRC-1 mediated HER2 Regulation in Endocrine Resistance

4.3.1 SRC-1 is not a limiting factor in resistant LCC9 cells

The proposed model for HER2 regulation suggests that in LCC9 cells oestrogen does not suppress HER2 mRNA and protein levels as SRC-1 is not as readily recruited to ERα as in MCF-7 and LCC1 thus being free to bind to the HER2 enhancer.

This implies SRC-1 is a limiting factor in MCF-7 and LCC1 cells. Reducing the amount of SRC-1 should mimic oestrogen action and help establish whether oestrogen-mediated HER2 repression is lost in LCC9 simply due to higher SRC-1 availability.

SRC-1 RNAi does not restore HER2 protein repression by oestrogen in LCC9 cells (Figure 4.11), suggesting that this mechanism is not only dependent on SRC-1 availability. These results mirror the conclusions of the study by Naughton *et al*, 2007 where transcription in LCC9 cells was found to be independent of p160 co-activators.

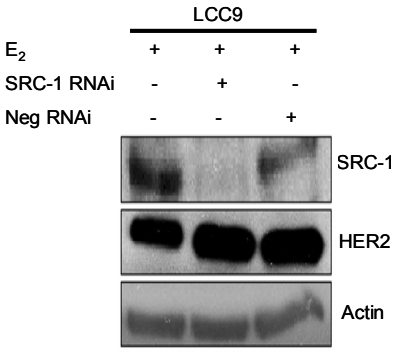


Figure 4.11 SRC-1 RNAi does not restore oestrogen mediated HER2 repression in LCC9 cells. Western blot analysis following treatment with SRC-1 siRNA. Results representative of four independent experiments.

4.3.2 SRC-1 RNAi mimics E_2 downregulation of HER2 in MCF-7 cells

The above results showing SRC-1 RNAi does not restore oestrogen mediated HER2 transcriptional repression in LCC9 cells highlight that this cofactor may not be as important as first proposed. It was therefore crucial to determine the precise role of SRC-1 in MCF-7 and LCC1 cells. According to the model, SRC-1 is sequestered by ERα after oestrogen treatment in these cells hence effectively the amount of SRC-1 protein available is reduced.

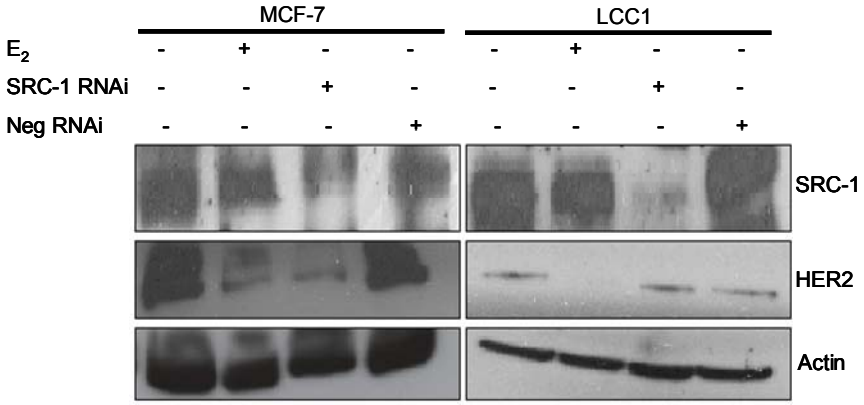


Figure 4.12 SRC-1 RNAi mimics oestrogen downregulation of HER2 in endocrine sensitive MCF-7 cells. Western blot analysis following treatment with SRC-1 RNAi in the absence of oestrogen. Results representative of three independent experiments.

If the availability of SRC-1 is the determining factor in regulating HER2 transcription of MCF-7 and LCC1 cells then oestrogen treatment and SRC-1 RNAi should produce similar results. SRC-1 RNAi treatment in the absence of oestrogenic stimulus mimics oestrogen treatment in MCF-7 cells (Figure 4.12). This however does not appear to be the case in LCC1 cells. This data suggests that the proposed mechanism for HER2 regulation appears to apply to MCF-7 and in part to LCC1 cells. The involvement of other p160 family members in the resistant cell line was considered next.

4.4 p160 Co-activators & their Role in HER2 Regulation

4.4.1 p160 Co-activators & Endocrine Resistance

The well characterized coactivator family consists of three members: SRC-1, TIF2 and AIB-1. These cofactors bind to the receptors in a ligand dependent manner and increase the receptors ability to activate gene transcription (McKenna et al, 1999).

The expression levels of the three p160 co-activators were previously measured in this cell line model (Naughton *et al*, 2007). SRC-1 and TIF2 protein levels are similar in MCF-7 and LCC1 cells. LCC9 cells, on the other hand appear to have lower SRC-1 and TIF2 expression in comparison to the other two cell lines. AIB1 protein expression is lower in LCC1 and lower still in LCC9 in relation to parental MCF-7 cells (Naughton *et al*, 2007). These results indicate that endocrine resistant LCC9 cells consistently express lower levels of p160 co-activators than endocrine sensitive cells.

Protein expression of p160 co-activators appears to differ between endocrine sensitive and endocrine resistant cell lines suggesting their role may be altered in LCC9 cells. Previous studies within our laboratory have determined the effects of SRC-1, TIF2 and AIB1 RNAis on cellular growth of MCF-7, LCC1 and LCC9 cells (Naughton *et al*, 2007). They demonstrated that all three co-activator RNAis induced similar growth responses therefore the data was presented as the mean of all cofactor RNAis. Cell proliferation is significantly reduced after siRNA treatment in MCF-7 and LCC1 whilst the growth of LCC9 cells remains unaffected. These results suggest that cofactor requirement and expression is progressively lost in this three stage model for acquired resistance (Naughton *et al*, 2007).

4.4.2 Role of TIF2 & AIB1

Previous results have shown that SRC-1 is important in regulating HER2 transcription of MCF-7 cells but control of this mechanisms appears to change in oestrogen independent LCC1 and be effectively lost in resistant LCC9 cells. The p160 family as mentioned above share a number of similar structures and functional domains therefore it was important to determine the role of the other two members of this family, TIF2 and AIB1.

4.4.2.1 TIF2 & AIB1 RNAis do not mimic E_2 in downregulating HER2

As previously shown SRC-1 RNAi mimics oestrogen in downregulating HER2 protein expression in MCF-7 cells. HER2 protein expression following TIF2 (Figure 4.13a) or AIB1 RNAi (Figure 4.13b) is not reduced as observed in the presence of oestrogen and is comparable to the control levels. Therefore, unlike SRC-1 RNAi, TIF2 and AIB1 RNAi treatment in the absence of oestrogen does not mimic oestrogenic repression of HER2 in MCF-7 cells. This implies that this mechanism is dependent on SRC-1 activity but does not require TIF2 or AIB1 expression in MCF-7 or LCC1 cells.

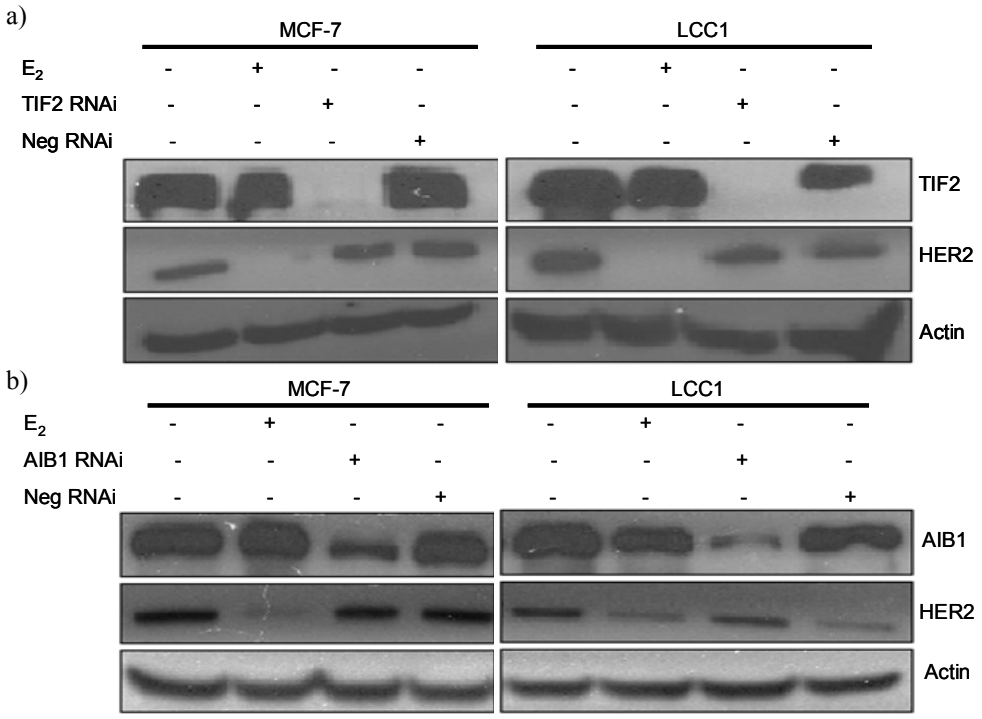


Figure 4.13 TIF2 & AIB1 RNAis do not mimic oestrogen in downregulating HER2 expression. Western blot analysis following treatment with TIF2 (a) & AIB1 (b) RNAis in the absence of oestrogen. Results representative of five independent experiments.

4.4.2.2 TIF2 & AIB1 RNAis: limiting factors in LCC9 cells

It was first proposed that oestrogen mediated HER2 repression was not observed in LCC9 cells since ER did not sequester SRC-1 leaving this cofactor free to bind to the enhancer region of HER2. According to this theory, HER2 transcription in LCC9 cells is unaffected as a result of unchanged SRC-1 availability. Yet mimicking oestrogen mediated SRC-1 reduction by treating LCC9 cells with SRC-1 RNAi did not restore HER2 repression suggesting that control of this mechanism is perhaps altered in these cells.

One possible explanation may include the remaining members of the p160 coactivator family as they have been shown to be highly homologous to SRC-1 (Nilsson et al, 2001). TIF2 and AIB1 did not restore HER2 repression after oestrogen treatment in LCC9 cells (Figure 4.14). This suggests p160 co-activators cannot individually restore oestrogen mediated HER2 downregulation in resistant LCC9 cells.

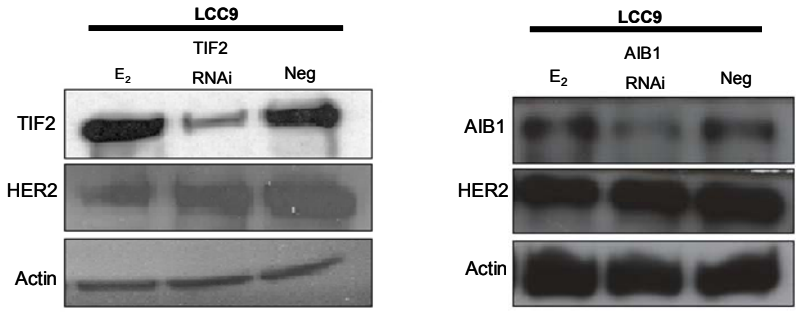


Figure 4.14 TIF2 & AIB1 RNAis do not restore oestrogen-mediated repression in resistant LCC9 cells. Western blot analysis following treatment with TIF2 (a) & AIB1 (b) RNAis in the presence of oestrogen. Results representative of five independent experiments.

4.5 Redundancy & p160 Co-activator Family

The p160 co-activators have similar structures and functional domains so it is not surprising that several studies have shown some level of redundancy between the members of this family (Nilsson et al, 2001; McKenna et al, 1999; Xu et al, 2003). Consequently it was important to consider redundancy in the mechanism being studied here. Individual coactivator RNAis are unable to restore oestrogen mediated HER2 repression in LCC9 cells however redundancy between the members of this family may account for the non effect. Double coactivator knockdowns should shed some light into role of this family in HER regulation.

4.5.1 SRC-1/TIF2 Double RNAi

SRC-1 and TIF2 RNAis are efficient in significantly reducing protein expression. SRC-1/TIF2 RNAi did not restore oestrogen mediated HER2 repression in LCC9 cells as HER2 mRNA expression is unchanged between RNAi and negative (Figure 4.15). These experiments were performed in the presence of oestrogen including control samples. In MCF-7 and LCC1 cells, SRC-1/TIF2 RNAi does not appear to have a significant effect on HER expression (Figure 4.15). It is important to note that these double knockout experiments were performed in the presence of oestrogen. This could account for the absence of HER2 transcriptional repression in MCF-7 cells following treatment with SRC-1 RNAi in Figure 4.15

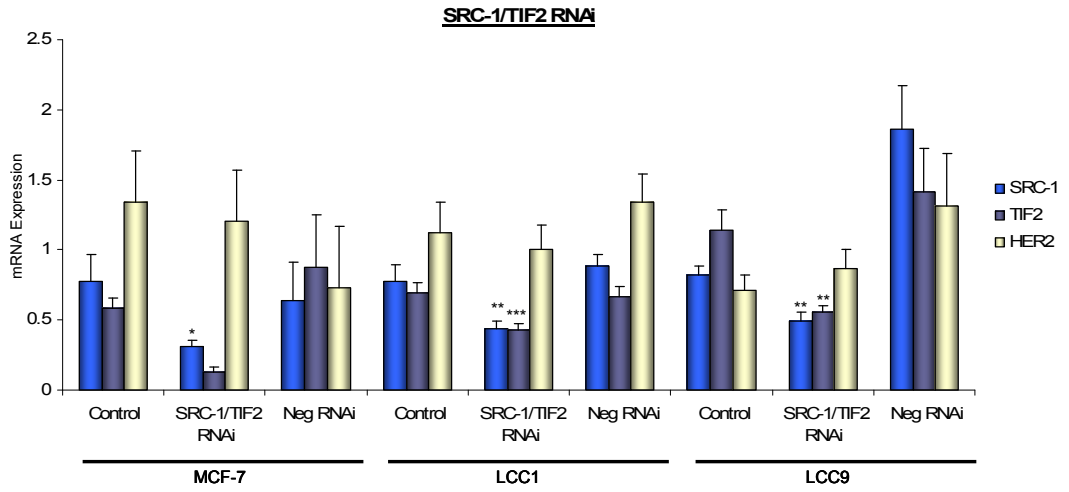


Figure 4.15 SRC-1/TIF2 double RNAi does not restore oestrogen mediated HER2 repression in LCC9 cells. RNAi efficiency and effects on HER2 expression were measured by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for each treatment vs matched negative control (Student's paired t-test *P<0.05; **P<0.01; ***P<0.001).

4.5.2TIF2/AIB1 Double RNAi

AIB1/TIF2 double RNAi treatment also did not restore HER2 repression in LCC9 cells despite RNAis significantly reducing AIB1 and TIF2 mRNA expression (Figure 4.16). In MCF-7 and LCC1 cells, HER2 expression is not altered by AIB1/TIF2 RNAi (Figure 4.16) though it is important to bear in mind TIF2 RNAi does not appear to be particularly efficient in MCF-7 cells.

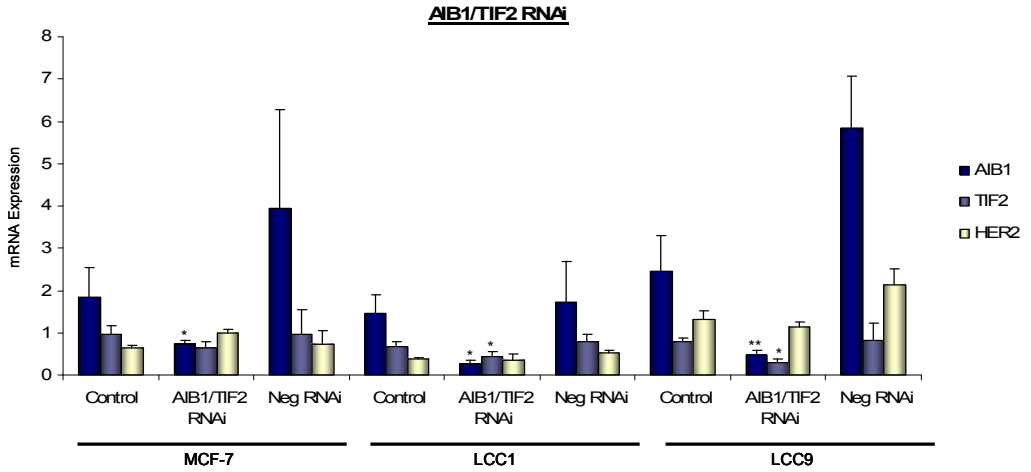


Figure 4.16 TIF2/AIB1 double RNAi does not restore oestrogen mediated HER2 repression in LCC9 cells. RNAi efficiency and effects on HER2 expression were measured by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for each treatment vs matched negative control (Student's paired t-test *P<0.05; **P<0.01).

4.5.3 SRC-1/AIB1 Double RNAi

SRC-1/AIB1 double knockdowns are the only pair to significantly reduce HER2 mRNA expression in LCC9 cells suggesting that AIB1 and SRC-1 may be equally important in regulating HER2 in these cells (Figure 4.17). This data also indicates that HER2 downregulation is achieved in LCC9 cells by interfering with co-activator expression hence the mechanism in these cells is in part regulated in a similar way to MCF-7 cells. Furthermore, HER2 expression was elevated in MCF-7 cells following treatment with SRC-1/AIB1 RNAi (Figure 4.17). This appears to also be the case in LCC1 cells though not significant which is likely to be a result of reduced protein knockdown by AIB1 RNAi.

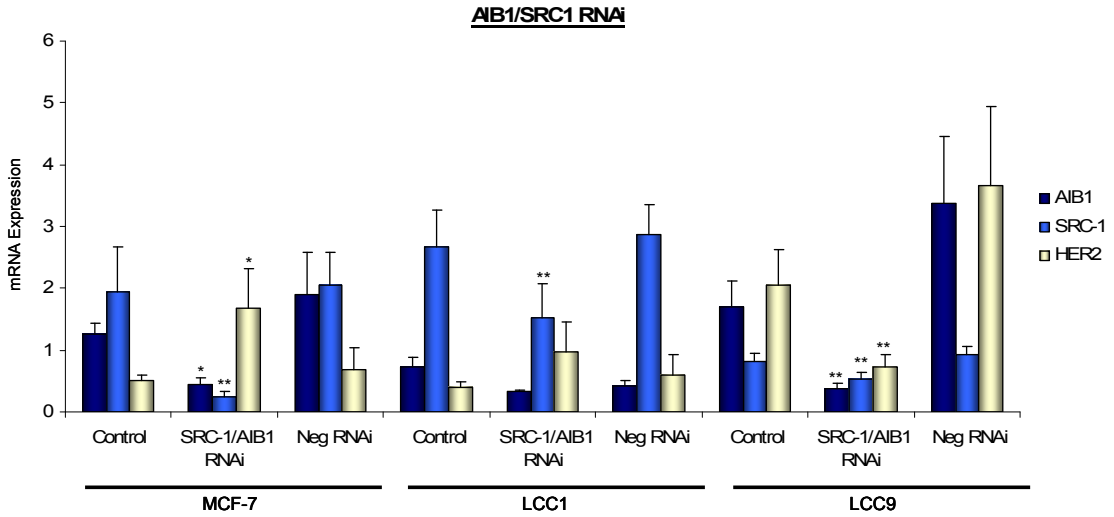


Figure 4.17 SRC-1/AIB1 double RNAi appears to restore oestrogen mediated HER2 repression in LCC9. RNAi efficiency and effects on HER2 expression were measured by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for each treatment vs matched negative control (Student's paired t-test *P<0.05; **P<0.01).

4.6 Discussion

Previous studies have suggested oestrogen potently inhibits HER2 transcription in ER-positive cell lines such as MCF-7 (Dati *et al*, 1990; Read *et al*,1990; Russell *et al*, 1992). The results shown in this Chapter suggest that altered regulation of this mechanism may be important in conferring resistance as oestrogen does not downregulate HER2 protein and mRNA expression in resistant LCC9 cells. In addition to HER2, oestrogen also downregulated HER3 and HER4 expression in MCF-7 and LCC1 cells, yet similarly to HER2, this was not observed in resistant LCC9 cells. Expression of EGFR, on the other hand, did not appear to be affected by oestrogen. HER2 amplification or enhanced expression has been previously associated with increased growth rate and poor prognosis of breast cancer tumours (Yu *et al*, 2000). Oestrogen-independent LCC1 and endocrine resistant LCC9 cells do not overexpress this receptor unlike other anti-oestrogen resistant cell line models (Knowlden *et al*, 2003; Jordan *et al*, 2004). However, deregulated transcriptional control of *HER2* may prove important in activating downstream signalling pathways which in turn may be play a part in conferring resistance.

The expression of ERα was found to be necessary and sufficient for oestrogen mediated HER2 transcription repression (Russell *et al*, 1992; Taverna *et al*, 1994). Studies also

found that the AF2 region of the oestrogen receptor was crucial in this regulatory mechanism as its removal effectively abrogated HER2 transcriptional inhibition. Furthermore, this ERα region is also important for p160 co-activators recruitment to the receptor ahead of transcriptional activity (Newman *et al*, 2000). Following these results a model was proposed whereby ERα and HER2 compete for p160 co-activator binding. Consequently, SRC-1 transfection was found to relieve oestrogen-mediated HER2 repression in ZR75-1 cells (Newman *et al*, 2000).

In line with this proposed model, ERα RNAi reversed the effects of oestrogen and restored HER2 protein expression back to basal levels in MCF-7 and LCC1 cells, even in the presence of 17β-oestradiol. Furthermore, SRC-1 RNAi alone mimicked oestrogen in downregulating HER2 protein expression suggesting this co-activator may be the limiting factor in MCF-7 cells. Previously published ChIP assays show that SRC-1 is not recruited to ERα in resistant LCC9 cells thus freeing SRC-1 to drive *HER2* transcription (Naughton *et al*, 2007). This suggested HER2 expression in LCC9 is not reduced by oestrogen due to elevated SRC-1 availability as this cofactor may not be recruited to ERα. However, SRC-1 depletion by RNAi did not restore oestrogen repression of HER2 expression indicating that in these cells SRC-1 may not be the limiting factor. The role of TIF2 and AIB1 was also determined but both these activators were found to not mimic oestrogen in the same way SRC-1 did and specific RNAi treatment also did not restored HER2 repression in the presence of oestrogenic stimulation.

Redundancy has often been described in this co-activator family and is particularly evident in mouse studies (Nilsson *et al*, 2001; McKenna *et al*, 1999; Xu *et al*, 2003). Given that no single cofactor restored oestrogen driven HER2 inhibition in resistant LCC9 it was important to establish by double knockouts whether redundancy was a factor. AIB1/SRC-1 double knockout significantly reduced *HER2* transcription suggesting that the enhancer sequence in this gene is still responsive to p160 co-activator regulation. It also indicates that AIB1 may be involved in this mechanism despite its individual knockdown having little effect. AIB1 expression is essential in HER2-driven oncogenesis in mice (Fereshteh *et al*, 2008) and is often upregulated in tamoxifen resistant human breast cancers (Anzick *et al*, 1997; Torres-Arzayus *et al*, 2004). Oestrogen independence in LCC1 and endocrine resistant in LCC9 are not associated with increased AIB1 expression as previously observed by our group (Naughton *et al*, 2007). In fact, these cells appear to express lower levels of this co-activator in comparison to the parental MCF-7 cell line.

A recent study by Hurtado *et al* (2009) have suggested that *HER2* transcription is tightly regulated by paired box 2 gene product (PAX2) whose exact function was yet to be determined. PAX2 siRNA treatment in MCF-7 and ZR75-1 cells abrogated oestrogen mediated inhibition of HER mRNA and lead to elevated HER2 protein expression. According to their study, AIB1 also regulates HER2 transcription by promoting HER2 transcription hence they suggest there is a stoichiometric balance between the repressor PAX2 and the co-activator AIB1 affecting binding and activation of *HER2* (Hurtado *et al*, 2009). This may explain why AIB1 siRNA in conjunction with SRC-1 siRNA reduced HER2 mRNA expression in LCC9 cells.

A series of studies have shed further light onto the mechanisms regulating *HER2* in mammary epithelial cells. A member of the forkhead/winged helix transcription factor family, the FOXP3 protein had been found to bind and repress the *HER2* promoter (Zuo *et al*, 2007). Downregulation of the *FOXP3* gene is commonly present in breast cancer tumours and directly correlates with HER2 expression (Karanikas *et al*, 2008; Zuo *et al*, 2007). In addition, the Ets transcription factor PEA3 has also been implicated in *HER2* inhibition (Hurst *et al*, 2001). PEA3 expression has been associated with HER2 status in primary breast tumour samples (Fleming *et al*, 2004) and with a decrease in *HER2*-driven tumourigenesis in breast cancer cell lines (Xing *et al*, 2000). Interestingly, PEA3 expression has been found to be associated with SRC-1 expression and tumours positive for both proteins were at a higher risk of recurrence (Fleming *et al*, 2004). These results provide a link between two proteins with opposing effect on *HER2* transcriptional regulation since SRC-1 is thought to promote HER2 expression whilst PEA3 inhibits this. Furthermore, increased levels of AP-2γ in relation to AP-2α expression was linked to oestrogen repression of HER2 despite both of them being equally effective activator of *HER2* promoter (Orso *et al*, 2004). This emphasises that *HER2* regulation may not only be dependent on certain proteins, it may also be determined by the stoichiometric balance between them.

These studies together with the results presented here suggest that transcriptional regulation of the *HER2* gene is mediated by a number of transcriptional factors (AP-2, PAX2, FOXP3 and PEA3) and co-regulators (SRC-1 and AIB1). These cofactors appear to promote *HER2* transcription whilst the transcription factors are mostly inhibitory. This

suggests that stoichiometric balance between these proteins may be important and that different activation/inhibition mechanisms may be regulated by distinct transcription factors or co-factors. In tamoxifen resistant cell lines overexpressing HER2, PAX2 expression is reduced whilst AIB1 expression is elevated (Hurtado *et al*, 2009). It is possible that a similar mechanism is at work in resistant LCC9 cells where no oestrogen-mediated *HER2* inhibition is observed. In our three-step MCF-7-based cell line model of endocrine resistance, SRC-1 and AIB1 cofactor expression are crucial in promoting *HER2* transcription. These factors may be displaced from the promoter or may be replaced by transcriptional inhibitors following oestrogen treatment thereby reducing HER2 expression. This may not be the case in resistant LCC9 cells hence HER2 expression remains unaffected. A summary of proteins involved in *HER2* transcriptional regulation is shown in Figure 4.18.

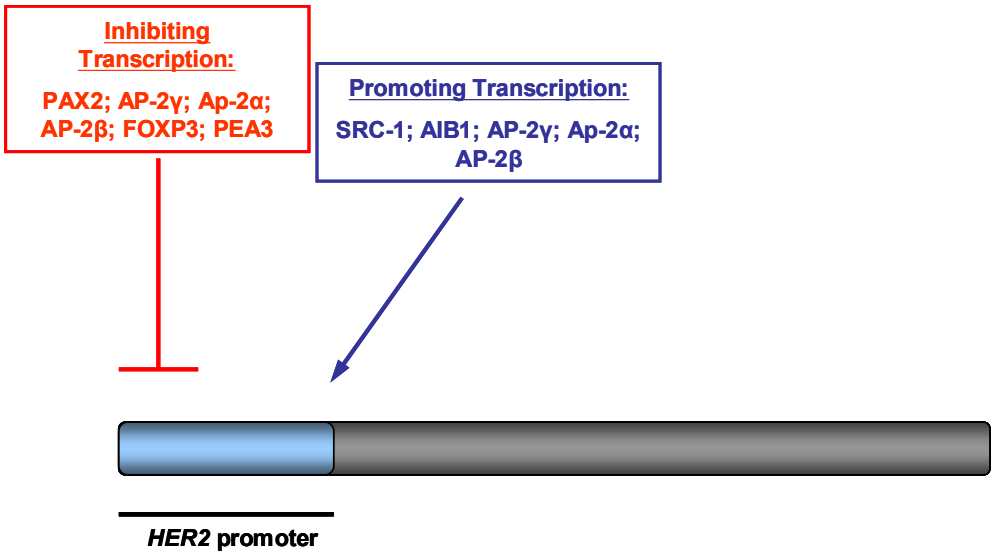


Figure 4.18 Summary of proteins involved in *HER2* transcriptional activation and inhibition.

Chapter 5

HER Family Regulation and p160 Co-activator Family: Analysis of Primary Tumour Material

Transcriptional regulation of the HER family members appears to be in part mediated by the p160 co-activator family as shown in Chapter 4. The data shown here and in previous studies (Newman et al, 2000) refers to work performed in breast cancer cell lines. Therefore, it was important to investigate further using primary tumour material. Breast carcinoma samples were kindly donated by Professor John Bartlett's laboratory (Edinburgh Cancer Research Centre) and have been extensively used in a number of different studies. (Kirkegaard et al, 2007; Kirkegaard et al, 2005; Cannings et al, 2007; Tovey et al, 2005; McGlynn et al, 2009).

Immunohistochemical staining was performed for SRC-1 as this co-activator appears to be the crucial member of the p160 family in terms of HER transcriptional regulation. These tissue microarrays (TMAs) have also been previously stained for AIB1 (Kirkegaard et al, 2007) and HER1, 2 & 3 (Tovey et al, 2005) thus it was possible to analyse SRC-1 expression in relation to these markers.

5.1 Clinical & Pathological Properties

A total of 402 breast carcinomas samples were collected between 1989 and 1999. Tissue microarrays were constructed containing triplicate samples of tumour areas selected from each individual block by a pathologist. These TMAs have been used in a variety of studies consequently reducing the number of samples available for SRC-1 immunohistochemical staining. Some cases were also excluded due to loss of core or the presence of insufficient tumour material. Therefore, not all of the 402 breast carcinoma samples were included in the analysis of SRC-1 staining. A total of 251 samples were available for SRC-1 staining. This series consisted of ERα-positive cases only. Patients were treated with tamoxifen for a median duration of 5 years (varying between 6 months and 18 years) and followed up for a median of 6.45 years. Clinical and pathological characteristics are described in Table 5.1.

	No./Total	%
Nodal Status		
0	119/251	47.3
1-3	65/251	25.9
4+	40/251	15.8
Grade		
1	57/251	22.7
2	112/251	44.5
3	26/251	23.8
Size (mm)		
T_1 (<20)	93/251	37
T_2 (20-50)	126/251	50.1
T_3 (>50)	13/251	5.1

Table 5.1 Clinical & Pathologic Tumour Variables. These are in reference to the tumours stained with the SRC-1 antibody only.

5.2 SRC-1 Protein Expression

SRC-1 protein expression was evaluated in 206 of 251 (82.1%) breast carcinomas. SRC-1 protein expression was confined to invasive breast carcinoma cells with no staining of normal breast epithelial cells (Figure 5.1). SRC-1 protein expression was primarily localised in the nuclei of tumour cells although weak staining was also observed in the cytoplasm. The median SRC-1 histoscore was 150. In subsequent analysis, SRC-1 histoscores above the median histoscore (upper quartile) were categorized as high expression whilst histoscores under the median were considered low (Figure 5.2).

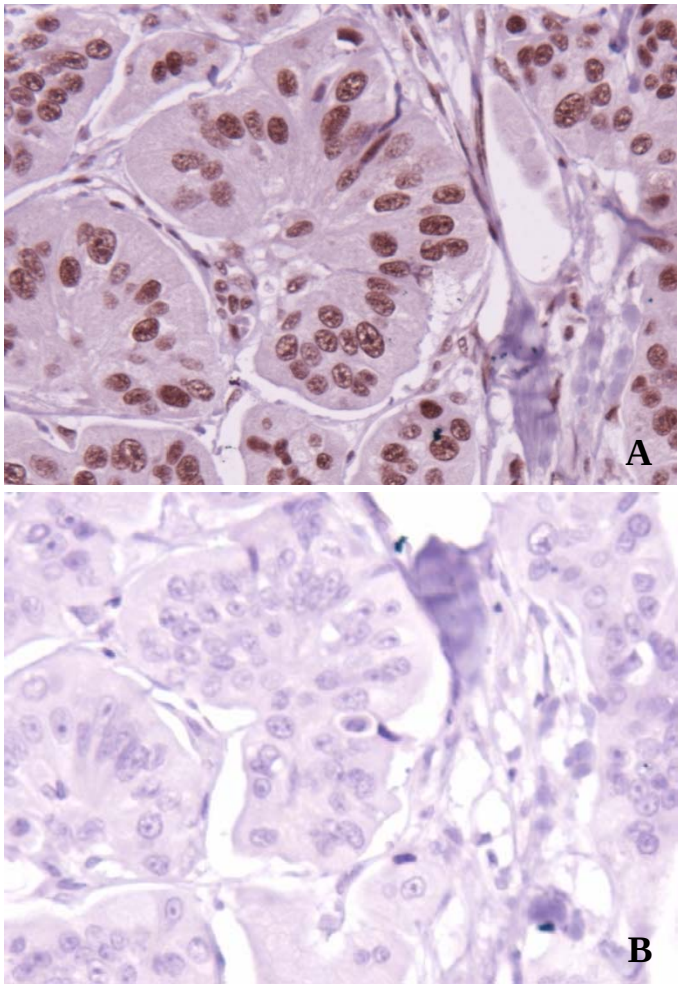


Figure 5.1 Photomicrographs of immunohistochemical staining in breast carcinomas with SRC-1 antibody. SRC-1 protein was mainly detected in the cellular nucleus (A). Breast tumours showed no staining in the absence of SRC-1 antibody (Negative Control) (B).

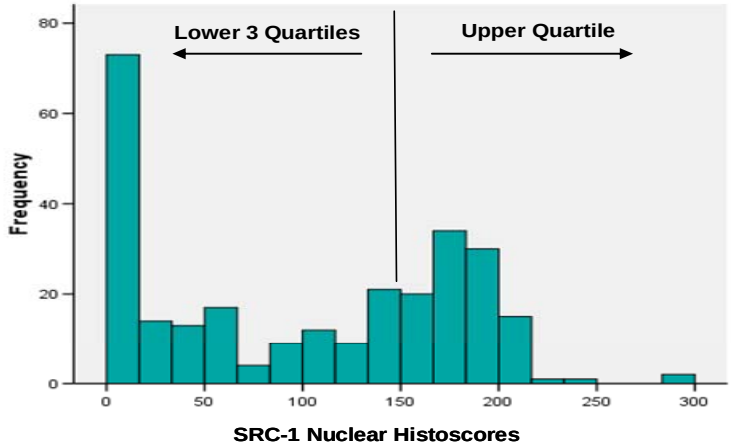


Figure 5.2 Histogram depicting distribution of SRC-1 histoscores in breast cancer patients used in this study.

5.3 SRC-1 Expression & Known Prognostic Factors

The expression of the SRC-1 co-activator was then compared to known prognostic factors such as nodal status, tumour grade and size. The Bloom-Richardson grading scheme was used to determine tumour grade in these breast carcinomas (Meyer et al, 2005). This system takes into account three morphologic features: tumour tubule formation, mitotic activity and nuclear pleomorphism. The resulting scale is divided into three categories: low grade (*well differentiated tumour*), intermediate grade (*moderately differentiated tumour*) and high grade (*poorly differentiated tumour*). There appears to be no association between SRC-1 and tumour grade (Table 5.2).

The tumours were also categorized according to nodal status (number of positive nodes) into four categories: 0, 1-3 and 4+. Similarly to tumour grade, there appears to be no correlation between SRC-1 protein expression and nodal status (Table 5.2). Moreover, there was no association between tumour size (3 categories: <20 mm; 20-50 mm; >50 mm) and SRC-1 expression (Table 5.2).

	SRC-1 Positive		
	No. of Patients/Tumours	**% Within Subgroup**	***p value***
Tumour Grade			
I	17	24.6	0.683
II	31	23.8	0.688
III	20	29.4	0.524
Nodal Status			
0	19	19.99	0.179
1-3	42	29.6	0.092
4+	5	0.29	0.170
Tumour Size (nm)			
< 20	20	21.5	0.184
20-50	16	24.2	0.143
> 50	12	29.3	0.233

Table 5.2 Patient and tumour characteristics stratified by SRC-1 status.

5.4 SRC-1 Expression & Patient Outcome

The role of SRC-1 expression in regulating patients' response to tamoxifen and relapse was also determined. After 5 years tamoxifen treatment, patients expressing higher SRC-1 levels appear to have reduced response to treatment. This is only a trend as the p-value is far from reaching significance (*p=0.178*) (Figure 5.3a). Furthermore, SRC-1 expression had no effect at all on the overall survival of these patients (*p=0.367*) as can be observed by the fact that the two plots for high and low SRC-1 expression are practically superimposed (Figure 5.3b).

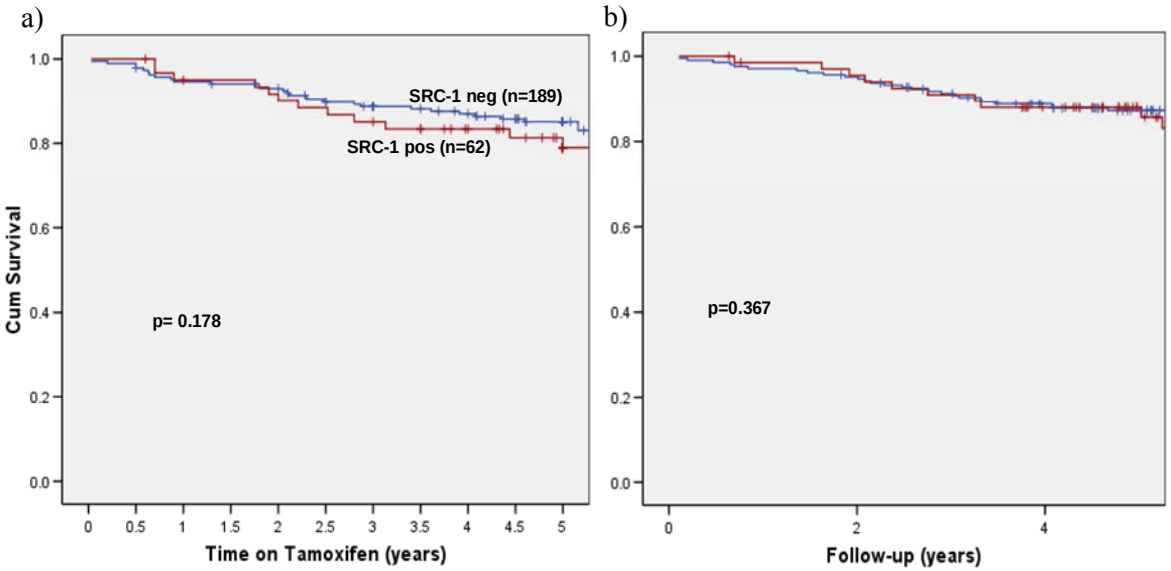


Figure 5.3 Kaplan-Meier Survival Curves showing cumulative disease-free survival between SRC-1 positive and negative patients. (a) Cumulative disease-free survival in this case refers to breast cancer disease relapse whilst on tamoxifen. (b) Breast Cancer relapse cases in relation to follow-up (years). P-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

5.5 Association between SRC-1 and AIB1 Expression

As previously published (Kirkegaard *et al*, 2007), high AIB1 expression was linked to relapse whilst on tamoxifen treatment. Once again this appears to be just a trend as the p-value fails to reach significance (*p-value=0.141*) (Figure 5.4a). On the other hand, high AIB1 expression is significantly associated with reduced survival (*p-value=0.012*) as previously published (Figure 5.4b).

Following evidence that AIB1 may play a part in patient outcome, the expression of this co-activator was compared to SRC-1 expression. There was no direct correlation between SRC-1 and AIB1 expression (*p=0.519*). Patients with high levels of SRC-1 and AIB1

expression appear to be more likely to relapse whilst on tamoxifen treatment. However, this is not significant (*p-value=0.124*) (Figure 5.5a).

With regards to overall survival high SRC-1/AIB1 expression appears to be linked to lower survival (*p-value=0.036*). This value is most probably significant due to the effect of AIB1 expression as SRC-1 alone did not alter survival ability (Figure 5.5B).

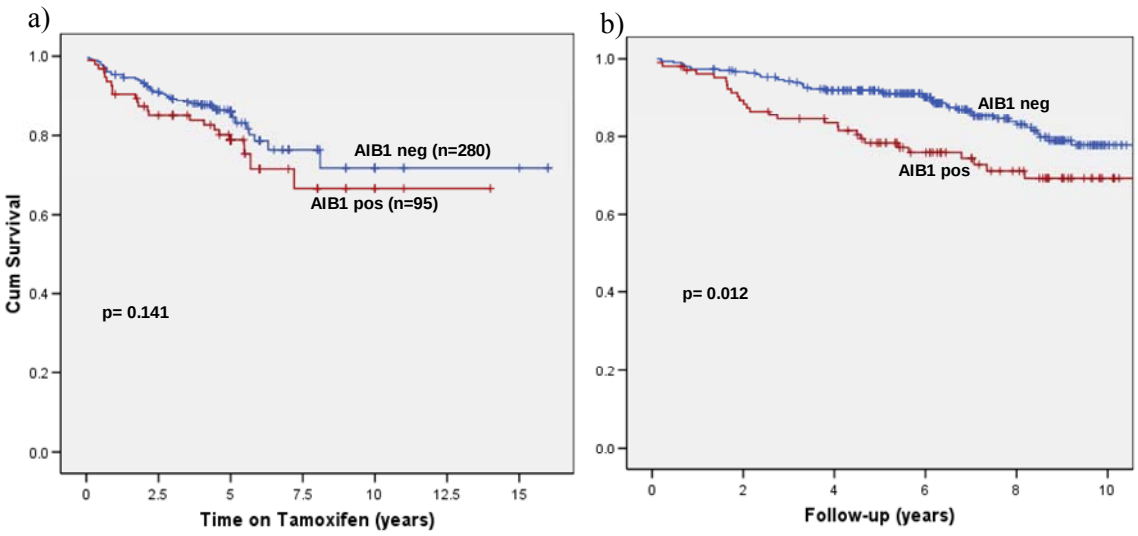


Figure 5.4 Kaplan-Meier Survival Curves were plotted to determine the effects of AIB1 positivity in breast cancer patients. Cumulative disease-free survival in reference to time on tamoxifen treatment (a) and follow-up (years) (b). P-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

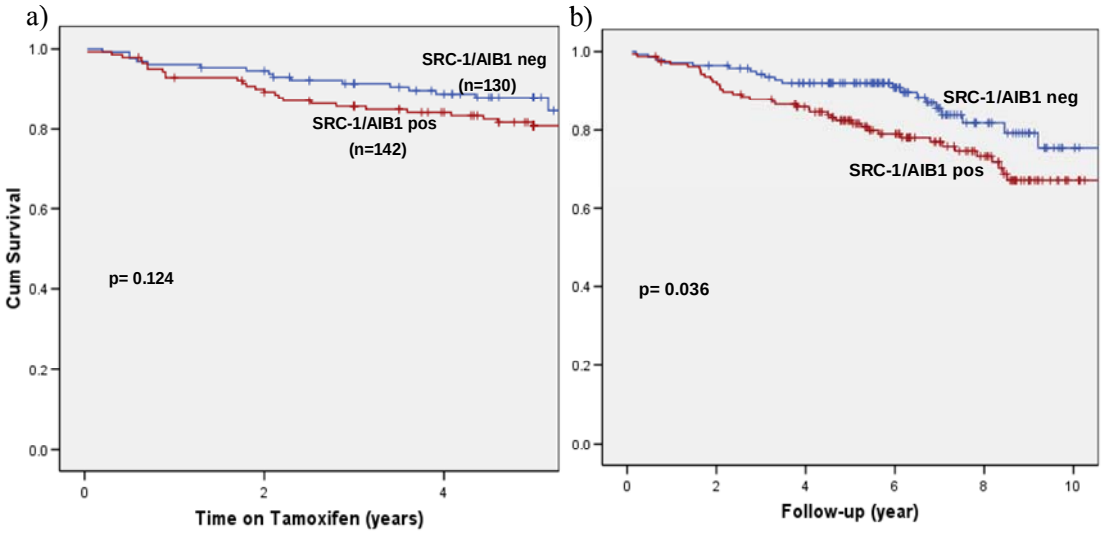


Figure 5.5 Kaplan-Meier Survival Curves demonstrating cumulative disease-free survival differences between patients positive and negative for SRC-1 & AIB1. As described before, cumulative disease-free survival was established in relation to time on tamoxifen treatment (a) and follow-up (year) (b). P-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

5.6 p160 Coactivator & HER Receptor Expression

As shown by Tovey *et al* (2005), patients expressing more than one HER family member are linked to increased relapse on tamoxifen and reduced survival (Figure 5.6). Furthermore as shown in Chapter 4, SRC-1 may be important in regulating HER receptor expression hence it was important to determine the relationship between receptors and SRC-1 expression. There appears to be no correlation between SRC-1 & AIB1 expression and HER2 amplification (*p-values=1 & 0.129 respectively*). Moreover, the expression of SRC-1 or AIB1 did not correlate with the expression of at least one of the HER1-3 membrane receptors (*p-values=0.156 & 0.129 respectively).*

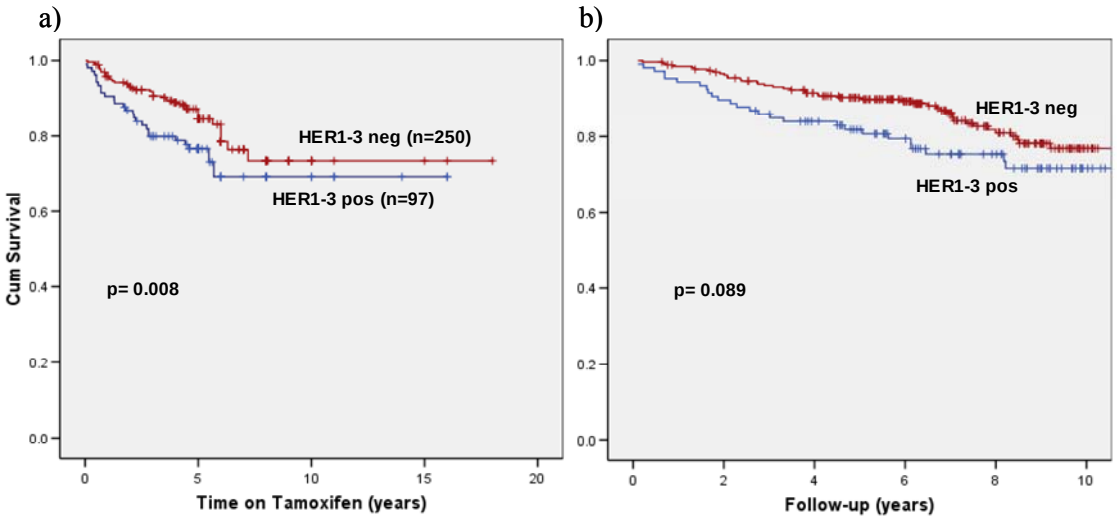


Figure 5.6 HER receptor expression and response to tamoxifen (a)**/ overall survival** (b)**.** Kaplan-Meier survival curves determining cumulative disease-free survival differences between patients positive and negative for Her1-3 (positive for one of the receptors). P-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

The effects of high HER receptor and co-activator expression on tamoxifen response and overall survival were also assessed. Overexpression of SRC-1 or AIB1 in HER1-3 positive tumours did not significantly affect patients' response to tamoxifen treatment (*p-value=0.473 & 0.573 for SRC-1 and AIB1 respectively*) though SRC-1/HER1-3 overexpression appears to have a protective effect against tamoxifen relapse for the first 5 years of treatment (Figure 5.7a/b). SRC-1 and AIB1 overexpression in HER1-3 positive tumours also did not affect the overall survival in relation to follow-up (years) (*p-values= 0.979 & 0.509*) (Figure 5.8 a/b).

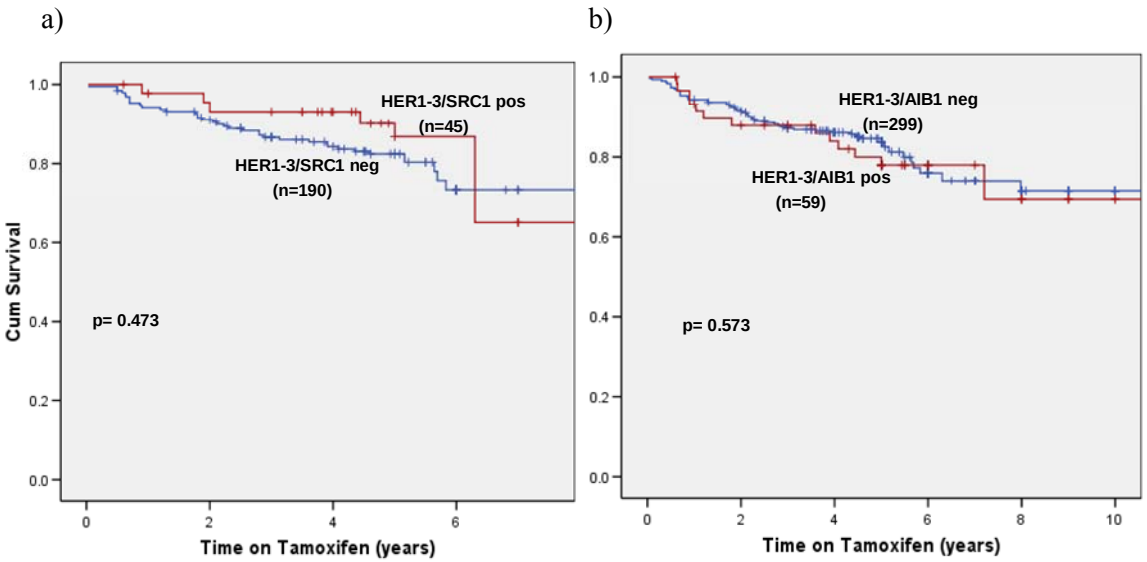


Figure 5.7 Kaplan-Meier survival curves determining cumulative disease-free survival differences (vs time on tamoxifen) between SRC-1/HER 1-3 (A) & AIB1/HER 1-3 (B) overexpressing tumours in relation to other tumours. Negative in this case refers to SRC-1 staining. p-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

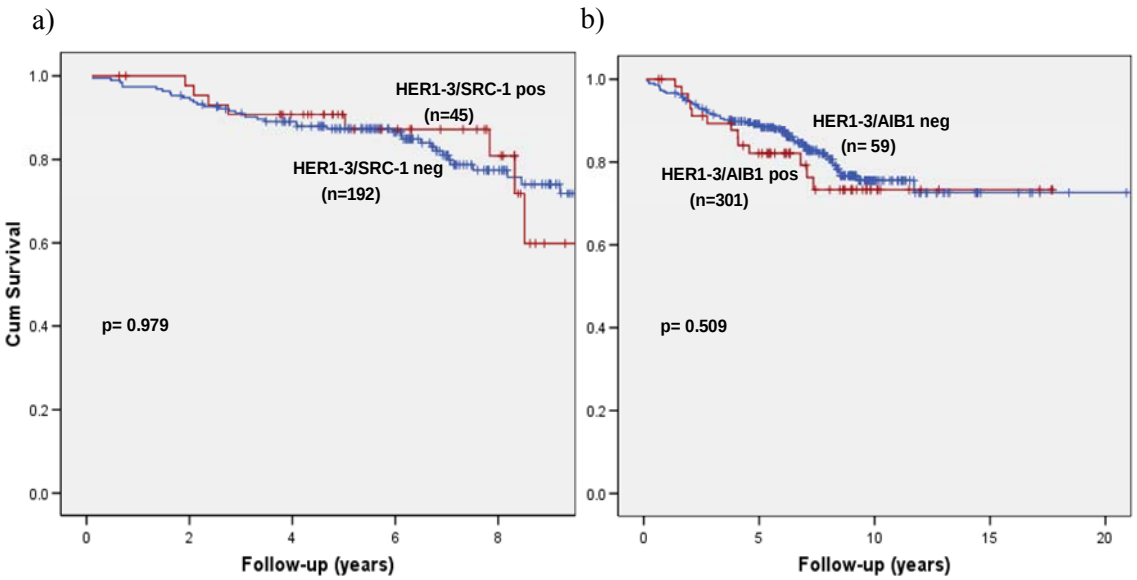


Figure 5.8 Kaplan-Meier survival curves determining cumulative disease-free survival differences (vs follow-up (years)) between SRC-1/HER 1-3 (A) & AIB1/HER 1-3 (B) overexpressing tumours in relation to other tumours. Negative in this case refers to SRC-1 staining. p-values obtained by log-rank testing for differences in cumulative disease-free survival between the two groups.

5.7 Discussion

As shown in Chapter 4, transcriptional regulation of the epidermal growth factor receptor family is altered in endocrine resistant cell lines. This mechanism appears to be mediated by p160 co-activators, particularly SRC-1 and AIB1. Oestrogen inhibits p160 co-activator mediated transcription of HER2, HER3 and HER4 receptors in endocrine sensitive cells however this is not observed in resistant LCC9 cells. In the presence of oestrogenic stimulation, SRC-1 preferentially binds to ERα hence it is not available to stimulate receptor transcription. This is not observed in the endocrine resistant cell line.

The results obtained in the MCF-7, LCC1 and LCC9 cells suggest there may be a direct link between p160 co-activators and epidermal growth factor receptor expression. Furthermore, it also highlights that this mechanisms may be important in conferring endocrine resistance. In order to investigate this further, breast cancer patient tissue was used thereby giving a clinical perspective. Previous studies (Newman *et al*, 2000) together with the results presented in Chapter 4 have shown SRC-1 co-activator is of particular interest hence this protein was the focus of immunohistochemical analysis. The breast carcinoma samples donated by Professor John Bartlett have been previously used in a variety of studies and have been immunohistochemically stained for AIB1 and HER1/ 2/3. Consequently, it was possible to compare the SRC-1 results with the previously published results.

There appears to be no association between SRC-1 and prognostic factors such as tumour grade, tumour size and nodal status. This is in contrast to previous reports which suggest that SRC-1 is linked to nodal positivity (Fleming *et al*, 2004) and reduced tumour size (Green *et al*, 2008). Furthermore, elevated SRC-1 expression following 5 year tamoxifen treatment was present in patients with reduced response rates (not significant) but did not affect overall patient survival. Recent studies have reported conflicting results in relation to SRC-1 and patient survival. Redmond *et al* (2009) report that SRC-1 is a strong predictor of reduced disease-free survival whilst Green *et al* (2008) suggest high SRC-1 expression is a marker for longer overall and disease-free survival. However, most of the available literature supports the view that SRC-1 expression is negatively associated with overall survival and directly associated with disease recurrence and endocrine resistance (Scott *et al*, 2007; Myers *et al*, 2004; Fleming *et al*, 2004; Myers *et al*, 1998). Despite not achieving statistical significance, our results support this view.

Previous immunohistochemistry analysis using AIB1 antibody in this breast cancer tumour microarray (Kirkegaard *et al*, 2007) has revealed no association between the AIB1 expression and relapse during tamoxifen treatment or overall survival. However, there does appear to be a trend suggesting that high AIB1 expression was associated with development of tamoxifen resistance despite not reaching statistical significance. The results show here appear to indicate that AIB1 expression is strongly associated with reduced disease-free survival ($p=0.012$). This is supported by a number studies indicating that AIB1 expression is elevated in mammary malignant tissue (Hudelist *et* al, 2003; Henke *et al*, 2004; List *et al*, 2001) and it is linked to short term disease free survival (Dihge *et al*, 2008). On the other hand, other groups report that AIB1 did not predict survival (Thorat *et al*, 2008), endocrine resistance (Murphy *et al*, 2002) nor is it linked to any clinico-pathological factors (Iwase *et al*, 2003).

The association between AIB1 and SRC-1 expression was also established. AIB1/SRC-1 positive tumours are not associated with relapse during tamoxifen treatment yet patients expressing high levels of the two isoforms do appear to have reduced disease-free survival. There was no significant association between expression of these two co-activators in contrast to the findings by Redmond *et al* (2009) which have recently observed that SRC-1 and AIB1 expressions are significantly linked. This is probably a reflection of higher numbers of tumours available in comparison to the study presented in this chapter.

The interaction of EGF receptor family and p160 co-activators was also investigated. As previously shown by Tovey *et al* (2005) HER1-3 positive breast tumours are more likely to become resistant to tamoxifen treatment. Conversely, HER1-3 expression did not affect overall patient survival. According to results presented in Chapter 4 there may be a correlation between p160 co-activator and HER1-3 expression. However, there was no correlation between AIB1/SRC-1 and HER1-3 expression. AIB1/HER1-3 and SRC-1/HER1-3 expressions did not alter tumour's response to tamoxifen treatment and overall disease-free survival. This is surprising considering is has been previously published that high AIB1 expression in tumours expressing one or more of HER1, HER2 or HER3 is linked to an increased risk of relapse whilst on tamoxifen treatment (Kirkegaard *et al*, 2008). This discrepancy is probably a reflection of the reduced number of tumours analysed in this study in comparison to the work performed by Kirkegaard *et al*, 2008. Furthermore, there appears to be no association between SRC-1/AIB1 expression and *HER2* amplification. These results

suggest there may not be such a straightforward link involving the EGF receptor family and p160 co-activators. Nevertheless, a positive correlation between SRC-1 and AIB1 expression and HER2 status has been observed by a number of different groups suggesting there may be a positive association between them (Bouras *et* al, 2001; Myers *et al*, 2004; Fleming *et al*, 2004; Thorat *et al*, 2008; Yamashita *et al*, 2008).

Chapter 6

Role of Akt in conferring resistance to endocrine therapies

6.1 Akt and Endocrine Resistance

Akt has been shown to play an important role in regulating cell proliferation (Kandel *et al*, 1999) and cell cycle (Liang *et al*, 2002) whilst also promoting cell survival (Nicholson *et al*, 2002). Elevated Akt protein expression and Akt gene amplification are often observed in human cancers such as gastric adenocarcinomas, pancreatic, ovarian and breast cancers (Shtilbans *et al*, 2008).

Akt activation is significantly associated with resistance to endocrine therapy and reduced disease-free survival (Tokunaga *et al*, 2006) and increased levels of phospho-Akt have been reported in Tamoxifen-resistant MCF-7 cells (Jordan *et al*, 2004). Furthermore, expression of constitutively active Akt protects against tamoxifen induced apoptosis (Campbell *et al*, 2001) and ionizing radiation (Liang *et al*, 2003).

The aim of this chapter was to determine the role of the PI3K/Akt pathway in the development of endocrine resistance in our cell line model.

6.1.1 Increased Akt phosphorylation in endocrine resistant cell lines

Following the previous reports suggesting Akt is involved in conferring resistance it was important to determine whether this pathway plays a part in the cell line models (MCF-7, LCC1 and LCC9) used in this project.

The expression levels of total Akt protein (antibody detects Akt 1, 2 and 3 isoforms) are not markedly different in the three cell lines (Figure 3.1). Akt is phosphorylated at two sites, threonine 308 (Thr308) and serine 473 (Ser473). Studies have shown that Ser473 may regulate phosphorylation of T308 and is required for full Akt activation (Scheid *et al*, 2002). Levels of Ser473 are elevated in LCC1 and LCC9 (Figure 6.1)

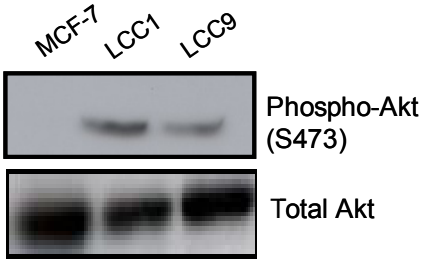


Figure 6.1. **Western Blot analysis of Akt phosphorylation** in parental MCF-7, oestrogen independent LCC1 and fully resistant LCC9 cells. Phospho-Akt antibody detects the three Akt isoforms. Results are representative of 4 separate

These results mirror the results published by Jordan *et al* (2004) and Yoo *et al* (2008) in different resistant cell line models.

6.2 Akt Pathway: Potential activation of downstream proteins

Elevated Akt phosphorylation is quite possibly an important mediator of acquired resistance in these cell line models however the precise mechanism needs to be determined.

6.2.1 Mammalian Target of Rapamycin (mTOR) & translational regulation

mTOR is phosphorylated by Akt following activation of the PI3K/Akt pathway and consequently activates the 40S ribosomal protein S6 kinase and inhibits the eukaryotic initiation factor 4E binding proteins (Hynes *et al*, 2006). This triggers enhanced function of the translational machinery therefore promoting cell growth (Wullschleger *et al*, 2006). Due to its location at the interface of a number of pathways mTOR is now a major target for the development of new cancer therapies.

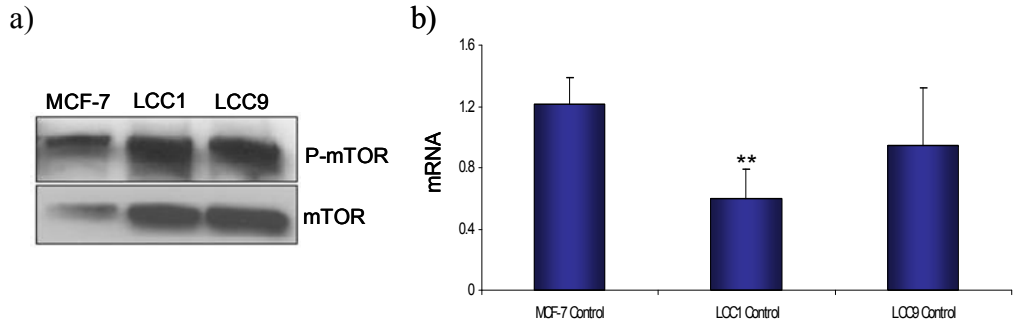


Figure 6.2. (a) **Western Blot Analysis of basal expression of mTOR and phospho-mTOR**. Results are representative of 3 independent experiments (b) Expression of mTOR mRNA in MCF-7, LCC1 and LCC9 by quantitative RT-PCR. Each column represents the mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for LCC1 compared with parental MCF-7 (Student's unpaired t-test **P<0.01).

The levels of mTOR mRNA are significantly lower in LCC1 (p-value=0.005) but not in LCC9 when compared to parental MCF-7 cell line (Figure 6.2b) however this does not seem to associate directly with the levels of total mTOR protein which are elevated in LCC1 and LCC9 cells (Figure 6.2a). It is therefore not surprising that phosphorylated mTOR (Ser2448) is also higher in these cells when compared to levels in MCF-7 cells (Figure 6.2a). It becomes difficult to determine whether the increased mTOR activation is a result of enhanced Akt phosphorylation or just a consequence of high total mTOR expression in LCC1 and LCC9 cells. These results also suggest that the increase in total

mTOR is not due to gene amplification or deregulated transcriptional activity since the mTOR mRNA levels are not higher in LCC1 and LCC9, and in fact the contrary is true. This suggests that the elevated mTOR protein expression may be due to changes at the translational level. High levels of phospho-mTOR in LCC1 and LCC9 suggests that translation may be enhanced in these cells therefore promoting growth.

One of the translational mediators activated by mTOR is the ribosomal S6 kinase which in turn phosphorylates 40S ribosomal protein S6 (Gingras *et al*, 2001). Levels of phospho-S6 (Ser371) are dramatically increased in LCC1 and LCC9 cells in comparison to parental MCF-7 (Figure 6.3).

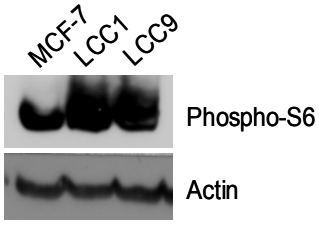


Figure 6.3 Western Blot analysis of phospho-S6 in MCF-7, LCC1 and LCC9 cells. Results representative of three independent experiments.

6.2.2 ERα Phosphorylation: Ser 167

Oestrogen receptors have two activation domains, the oestrogen dependent and oestrogen independent domains, the latter being often implicated in acquired resistance. Previous studies have shown MAPK phosphorylates ERα at Ser118 in the absence of oestrogen (Nilsson *et al*, 2001). This ligand independent activation of ERα is one route to conferring resistance to endocrine therapies.

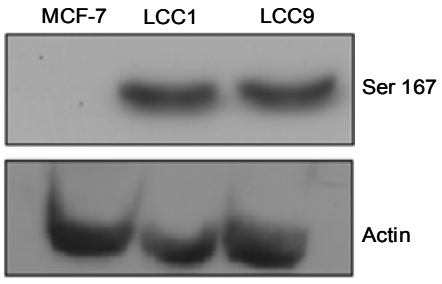


Figure 6.4 Western Blot analysis of ERα phosphorylation levels at two phosphorylation sites Serine 118 (Ser118) and Serine 167 (Ser167). Results representative of three independent experiments.

Campbell *et al* (2001) have shown that Akt also phosphorylates ERα at the oestrogen-independent activation domain (Ser167 residue). Phosphorylation of Ser167 is elevated in LCC1 and LCC9 cells. Ser 118 phosphorylation, on the other hand remains similar across the cell lines, perhaps even slightly reduced in LCC1 (Figure 6.4). The MAPK pathway is not altered in this model (as shown in Chapter 3) hence there is no change in Ser118 phosphorylation. Ser167 phosphorylation is effectively a downstream target of Akt as observed by Campbell *et al* (2001) so high levels of phospho-Ser167 are likely to be a result of elevated Akt phosphorylation.

6.3 Upstream Mediators of Akt Activation

Having established that in our endocrine resistant model Akt activation is elevated it was crucial to investigate which upstream pathways are involved in this increase in phospho-Akt.

6.3.1 PTEN Tumour Suppressor

Protein phosphatases are important in regulating Akt activity. PTEN negatively regulates PI3K by dephosphorylating its phosphoinositide products. Though PTEN mutations are relatively uncommon, PTEN protein loss due to promoter methylation or regulation at RNA/protein level is observed in 30% of breast cancers (Singh *et al*, 1998; Garcia *et al*, 2004).

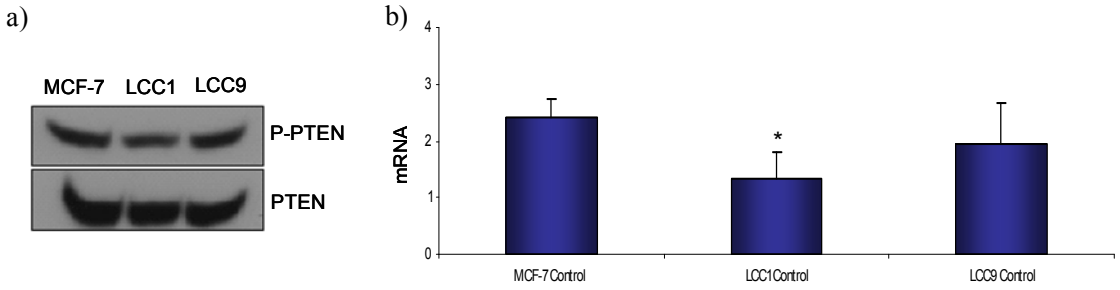


Figure 6.5 PTEN Expression a) Western blotting analysis of basal expression of PTEN and phospho-PTEN. Results representative of 3 separate experiments. b) Expression of PTEN mRNA in MCF-7, LCC1 and LCC9 by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for LCC1 compared with parental MCF-7 (Student's unpaired t-test *P<0.05).

At the mRNA level, LCC1 cells have significantly reduced PTEN mRNA levels in comparison to parental MCF-7 cells (p-value=0.014) (Figure 6.5b). This is not the case at the protein level since PTEN remains the same in all three cell lines (Figure 6.5a). This suggests that the increase in Akt phosphorylation is not due to loss of PTEN protein.

6.3.2 PI3K

Akt kinase activity is induced following PI3K activation by a number of growth-factor receptor-driven signalling cascades. Due to the link between PI3K and many cellular processes such as cell growth, proliferation and survival, it is not surprising that this kinase has been implicated in cancer (Franke *et al*, 2003).

The antibody used for these experiments recognises the p85 regulatory subunit. PI3Ks are heterodimeric molecules consisting of a p110 catalytic and a regulatory p85 subunit (Dillon *et al*, 2007). PI3K function requires the activation of both p85 and p110 subunits. Following receptor activation the regulatory p85 subunit is recruited and phosphorylated at the cell membrane which in turn activates the catalytic p110 subunit (Chang *et al*, 2003). Therefore activation of p85 is a marker for PI3K activation.

Phosphorylated PI3K is slightly elevated in LCC1 but certainly not in LCC9 where it is lower than in MCF-7 (Figure 6.6). p85 and thereby PI3K activation is not elevated in LCC1 or LCC9 cells suggesting that increased PI3K phosphorylation is not responsible for the high levels of phospho-Akt in these cells.

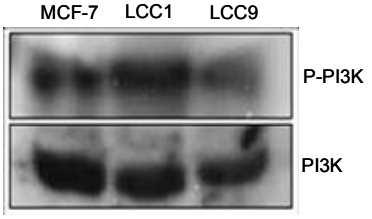


Figure 6.6 Western blotting analysis of PI3K p85 regulatory subunit. Result representative of three separate experiments.

Activating mutations in the gene encoding the catalytic subunit p110 α (PIK3CA) have been identified in a number of cancers, including breast cancer. A multiplexed assay developed by Board *et al* (2008) was used to detect PIK3CA mutations in this cell line model. This technique focuses on the most commonly reported mutations on exons 9 and 20 (H1047L; H1047R; E542K and E545K) (Levine *et al*, 2005) (Table 6.1). The exon 9 G1633A mutation was identified in MCF-7 cells as previously observed (Saal *et al*, 2005). LCC1 and LCC9 cells also carry the same mutation suggesting a mutational alteration is not occurring in this cell line model for endocrine resistance.

PIK3CA mutations in breast cancer cell lines				
Exon	**Nucleotide**	**Codon**	**Domain**	**Cell Lines**
9	G1624A	E542K	Helical	BT-483 (Bachman et al, 2004)
9	G1633A	E545K	Helical	**MCF-7, LCC1, LCC9**
20	A3140G	H1047R	Catalytic	T47-D (Bachman et al, 2004)
20	A3140C	H1047L	Catalytic	

Table 6.1 Most common PIK3CA mutations in breast cancer cell lines.

6.3.3 Insulin-Like Growth Factor Receptor (IGFR)

Altered IGFR function is frequently observed in breast cancer. IGFR signalling is known to activate PI3K and may therefore be responsible for elevated Akt phosphorylation in endocrine resistant cells. IGFR protein levels are similar in the three cell lines (Figure 6.7a) despite mRNA levels being greatly reduced in LCC1 and LCC9 (p-values 0.0001 and 0.001 respectively) (Figure 6.7b). Phospho-IGFR is elevated in LCC1 and LCC9 (Figure 6.7a) hence this pathway may be responsible for high phospho-Akt levels observed in these cells.

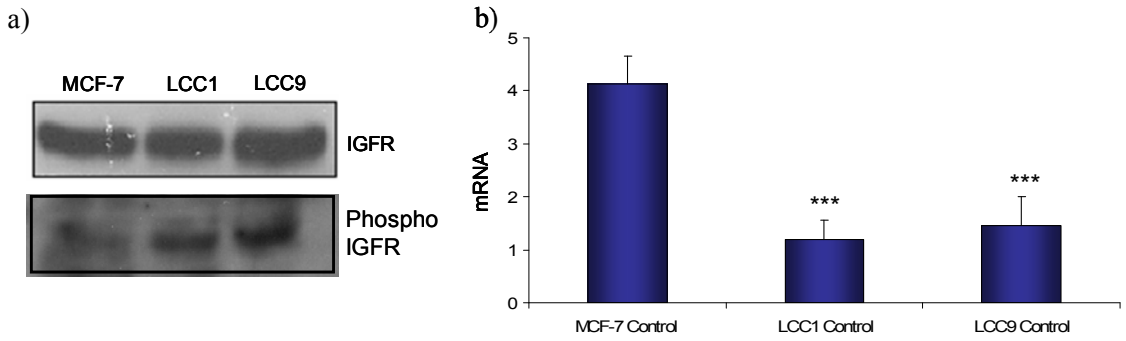


Figure 6.7 IGF Receptor Expression a) Western blotting analysis of basal expression of IGFR and Phospho-IGFR. Results representative of 3 separate experiments. b) Expression of IGFR mRNA in MCF-7, LCC1 and LCC9 by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for LCC1 and LCC9 when compared with parental MCF-7 (Student's unpaired t-test ***P<0.001).

The IGF signalling system is comprised of two ligands (IGFI and IGFII) which are significantly overexpressed in LCC1 and LCC9 cells when compared to the parental MCF-7 cell line (Figure 6.8). This is in agreement with previous studies that have shown increased IGFI expression in a number of breast cancer cell lines. The same study also reports that overexpression of IGFI and IGFII in MCF-7 leads to an increase in cell proliferation and tumour formation (Pacher *et al*, 2007).

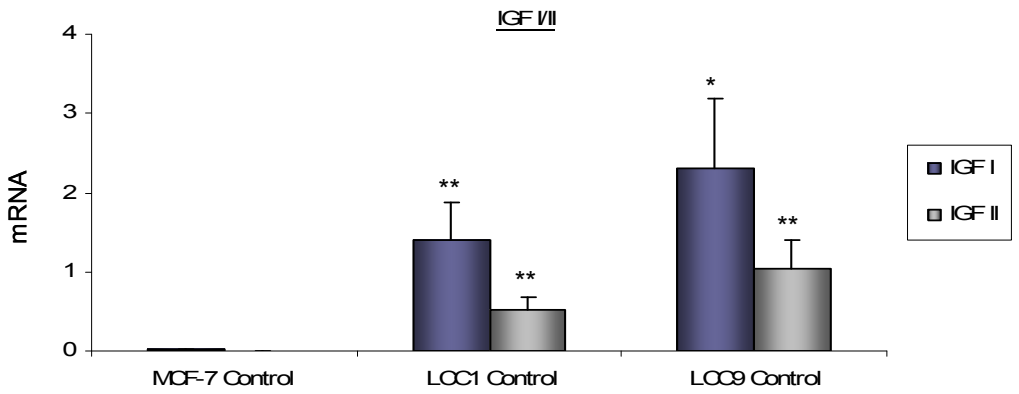


Figure 6.8 Expression of IGFR ligands IGF I and II mRNA in MCF-7, LCC1 and LCC9 by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance noted for LCC1 and LCC9 when compared with parental MCF-7 (Student's unpaired t-test *P<0.05;**P<0.01).

Following the above observations, an IGFR inhibitor was used to investigate the role of this pathway in acquired endocrine resistance. I-OMe AG538 inhibits IGFR autophosphorylation by competing with IGFR tyrosines (Nahta *et al*, 2005). The results show that this inhibitor did not reverse resistance to tamoxifen or ICI in LCC9. In the parental MCF-7 cells, I-OMe AG538 at 5 and 10 μM did significantly reduce growth (p-value 0.04 at both concentrations). Furthermore, combination of ICI and I-OMe AG538 also reduce growth in MCF-7 and LCC1, however this is probably due to the ICI effect rather than the IGFR inhibitor. LCC9 growth was not at all inhibited by the IGFR inhibitor.

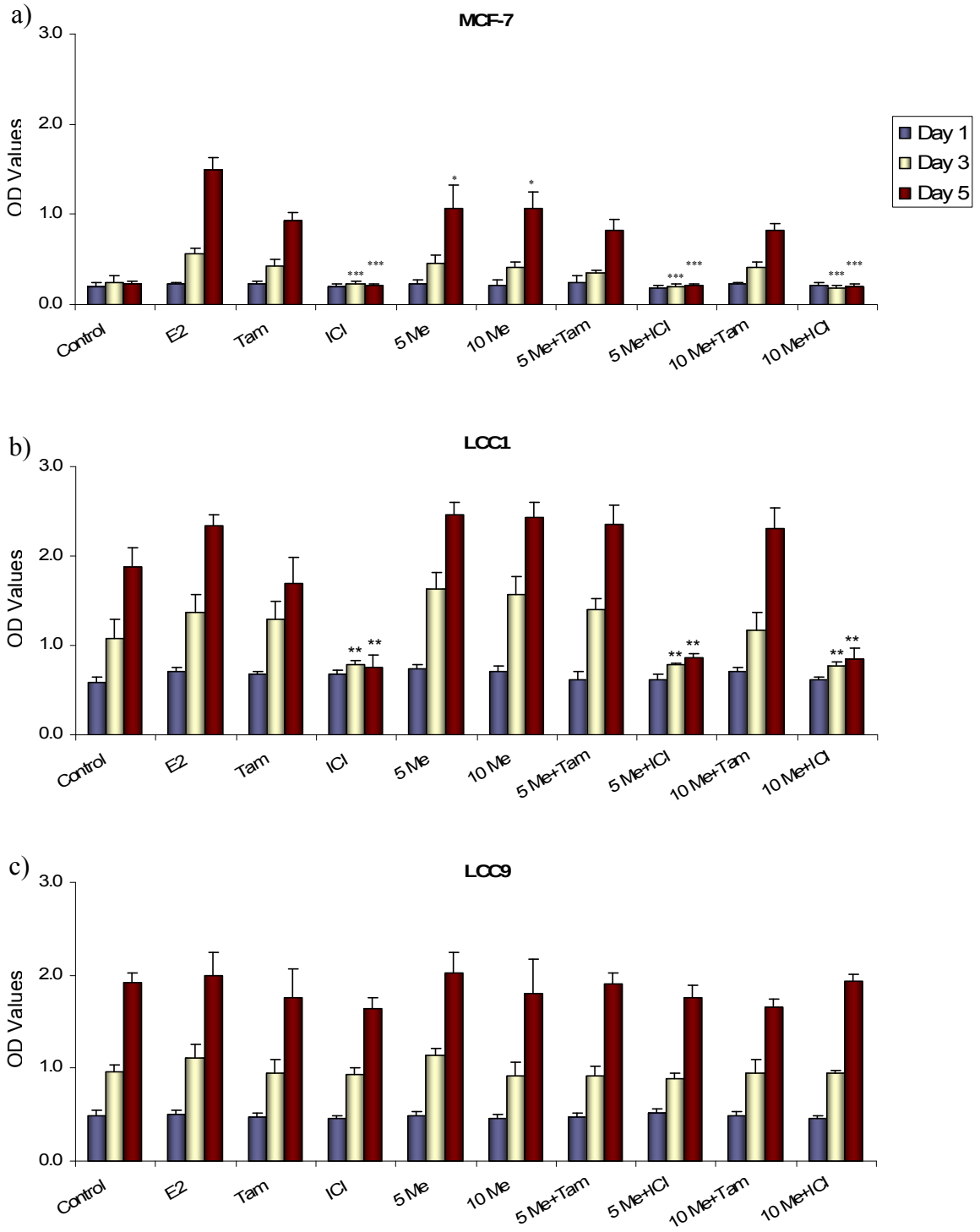


Figure 6.9 Effects of IGFR inhibitor I-OMe AG538 on the growth of MCF-7 (a), LCC1 (b) and LCC9 (c). MCF-7 experiments were conducted in the presence of E_2. Each column presents means of 6 OD values. Error Bars=SD. ANOVA test: statistical significance noted for ICI, 5Me, 10Me, 5Me+ICI and 10Me+ICI vs E treatment in MCF-7 and LCC1. *P<0.05; **P<0.01; ***P<0.001.

6.4 Akt Isoforms: Roles of Akt 1, 2 and 3

To date three Akt isoforms (Akt 1, 2 and 3) have been identified, all of which have been implicated in breast cancer (Jordan *et al*, 2004).

6.4.1 Basal Characterisation of Akt isoforms

Akt isoform activation has been linked to a variety of cancers. Elevated Akt 1 and 2 kinase activity has been reported in primary breast tumour material. In our cell line model, expression of Akt1 and 2 are significantly reduced in LCC1 (p-values=0.0001 & 0.013 respectively) and in LCC9 cells (p-values=0.002 & 0.009 respectively) in comparison to parental MCF-7 (Figure 6.10). On the other hand, Akt 3 mRNA expression is elevated in LCC9 cells only (p-value 0.015).

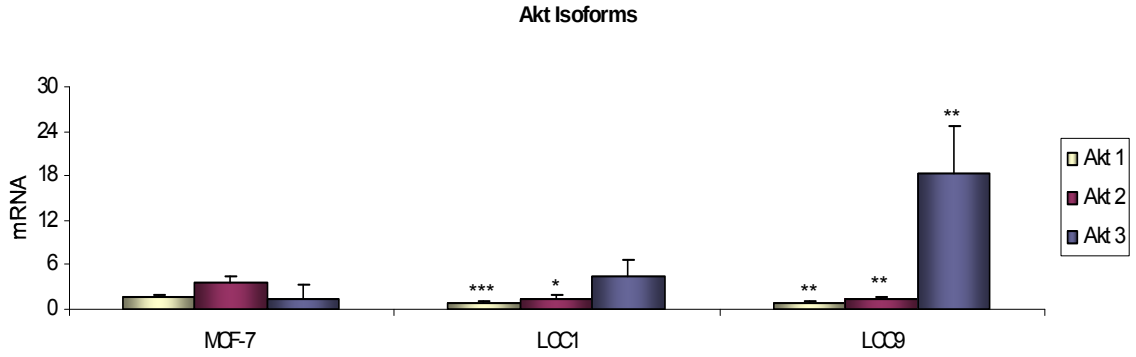


Figure 6.10 Expression of Akt isoform's mRNA in MCF-7, LCC1 and LCC9 by quantitative RT-PCR. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance tested between cell lines (MCF-7 Akt 1, LCC1 Akt 1 & LCC9 Akt 1; ANOVA test *P<0.05;**P<0.01; ***P<0.001).

MCF-7, LCC1 and LCC9 cells expressed equal levels of Akt 1 and Akt 2. Akt 3 protein expression, on the other hand is elevated in LCC1 and LCC9 cells (Figure 6.11). This is in contrast to the low expression of Akt3 expression in MCF-7 cells, a result that is supported by previous reports (Nakatani *et al*, 1999).

This data suggests that Akt3 expression is elevated in LCC9 due to increase transcription.

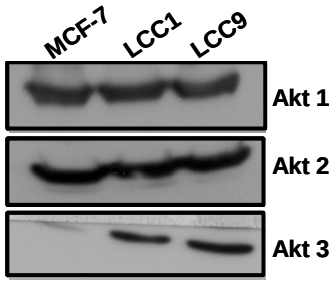


Figure 6.11 Western blotting analysis of Akt isoforms (Akt 1, 2 & 3). Result representative of three independent experiments.

6.4.2 Elevated Akt 2 Phosphorylation in Resistant LCC9 Cells

Unfortunately, there are no readily available phospho-Akt antibodies specific to each of the Akt isoforms. To overcome this, immunoprecipitation was performed using Akt isoform specific antibodies. The immunoprecipitates were then probed with anti-phospho-Akt antibody to reveal which isoforms are activated in each of the cell lines.

In the three cell lines, Akt 3 is highly phosphorylated (Figure 6.12). This is surprising particularly in MCF-7 since they have low Akt 3 expression. The level of Akt 3 phosphorylation becomes progressively higher in LCC1 and LCC9 in comparison to MCF-7. Perhaps more relevant is the striking difference in the levels of phospho-Akt2 between MCF-7 and the other cell lines. Phosphorylation of the Akt 2 isoform is high in LCC1 and LCC9. This is in contrast to the minimal phosphorylation levels observed in parental MCF-7 cells (Figure 6.12).

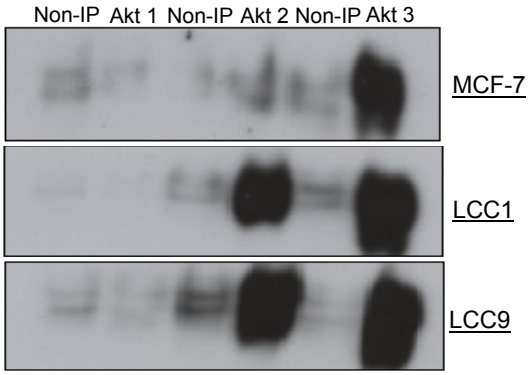


Figure 6.12 Phosphorylation of Akt isoforms. Samples were immunprecipitated with Akt 1, 2 & 3 specific antibodies. Western Blots were then performed using these lysates and probed for phospho-Akt. The non-IP control was incubated with IgG rather than isoform specific antibody. Result representative of 3 independent experiments.

The high levels of Akt 3 phosphorylation in MCF-7 cells is surprising considering Akt 3 basal levels in this cell line are very low as shown in Figure 6.11. As Akt 3 phosphorylation is present in all cell lines it is unlikely that this isoform is important in conferring resistance in this cell line model. On the other hand, Akt 2 phosphorylation may be of particular interest as it is only observed in oestrogen-independent LCC1 and endocrine resistant LCC9.

6.5 Functional Studies: Effects of Akt RNAi

6.5.1 Akt RNAi efficiency

Both Akt 1 and Akt 2 RNAis specifically reduced both RNA and protein levels of Akt 1 and Akt 2 isoforms, respectively (Figure 6.13a & b). However, Akt 3 RNAi appears to poorly reduce protein levels despite reducing mRNA levels as determined by quantitative

RT-PCR (Figure 6.13a & b). The figure shows the results obtained for LCC9 cells since these cells express high levels of all three Akt isoforms.

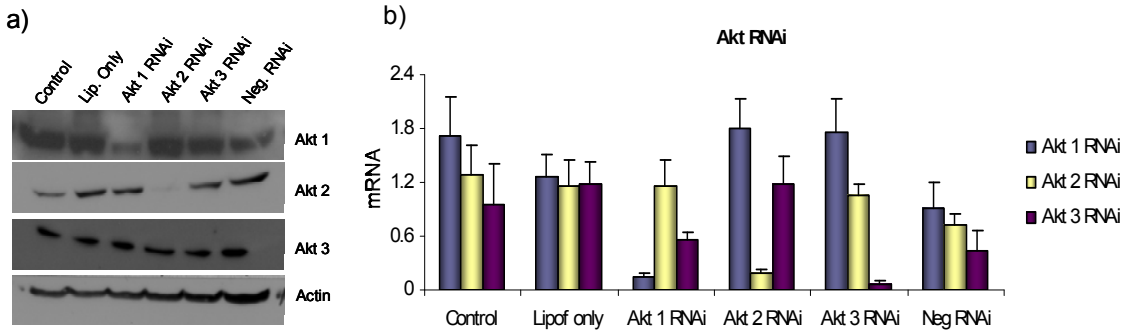


Figure 6.13 Akt RNAi efficiency and specificity in LCC9 cells analysed by western blotting (a) and quantitative RT-PCR (b) Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars= SD. Statistical significance tested between each Akt RNAi and appropriate negative (Student's Unpaired t-test *P<0.05;**P<0.01). All results shown are representative of three independent experiments

6.5.2 Effects of Akt RNAi on Growth

Parental MCF-7 cells were sensitive to Akt 1 and Akt 2 specific RNAis as cell number is dramatically reduced (Figure 6.14a). The number of viable cells at day 5 is 4 times lower in wells treated with these RNAis in comparison to negative RNAi treated wells. Akt 3 RNAi, on the other hand did not induce such a response. Taking into account the level of Akt 3 phosphorylation in all three cell lines, it would be expected that Akt 3 would yield the most dramatic effects which is not the case in MCF-7 (Figure 6.14a). This may be a reflection of little protein knockdown by Akt 3 RNAi. Interestingly, Akt 3 protein knockdown is more effective in LCC1 and LCC9 cells which, as shown in Figure 6.11, overexpress Akt 3 in comparison to parental MCF-7 cells. However, the lack of response to Akt 3 RNAi in MCF-7 cells does lead to questioning on how robust the immunoprecipitation results.

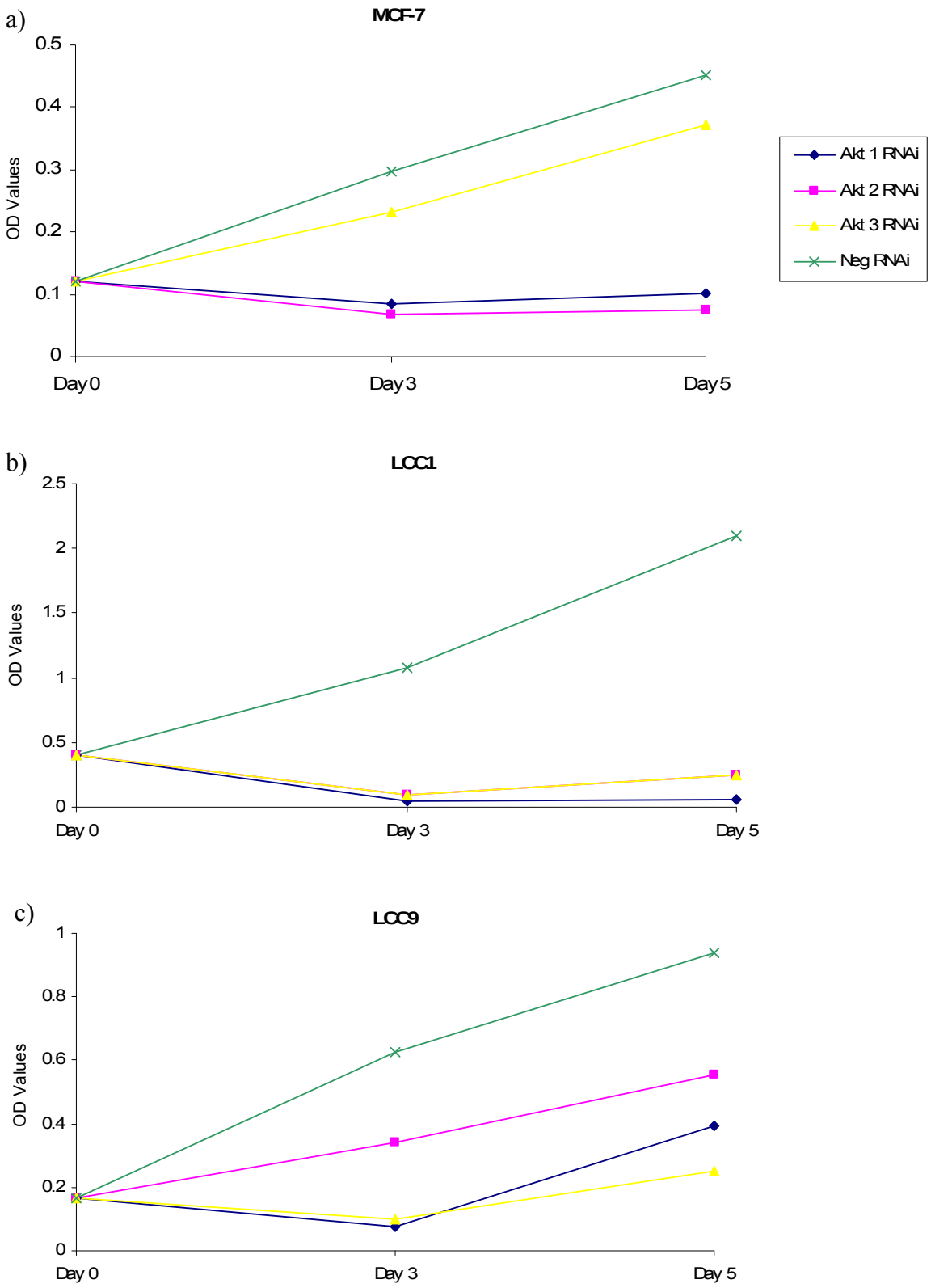


Figure 6.14 Effects of Akt 1, 2 & 3 RNAi on growth of MCF-7 (a), LCC1 (b) and LCC9 (c). MCF-7 experiments were conducted in the presence of E_2. Each point (Day 0, 3 & 5) presents the mean of 6 OD values. Negative RNAi is used as a negative control. This ensures that any variations observed are due to the RNAi effect and not down to the effects of the transfection process. Results representative of three independent experiments.

LCC1 cells are particularly sensitive to all three Akt RNAi as they all reduced growth (at least 8 times lower than control negative RNAi (Day 5 values taken into account)). Furthermore, in LCC1 cells Akt 3 RNAi appears to inhibit growth as efficiently as Akt 1 and Akt 2 RNAi (Figure 6.13b). Similarly to LCC1, LCC9 cellular growth is inhibited by all three Akt RNAis but not quite as dramatically as in LCC1 cells (Figure 6.14c). Furthermore, it is interesting to note that Akt 2 RNAi in LCC9 cells reduced growth but did not inhibit it to the same level of the other Akt RNAis. This is surprising considering previous results showing increased Akt 2 phosphorylation in LCC9 cells. Also interesting is the fact that LCC9 cells "recover" from Akt RNAi transfection much faster than any of the other cell lines. At Day 3, LCC9 cells begin to grow again whilst for MCF-7 and LCC1 cells growth inhibition is still observed after 5 days of treatment (Figure 6.14c).

These results suggest all three isoforms are important in the three cell lines, despite previous observations that Akt 2 and 3 are the prime targets for phosphorylation. Akt RNAis consistently reduced growth in endocrine sensitive and resistant cell lines suggesting that the role of these isoforms may be critical to cell growth.

6.5.3 Effects of Akt RNAi on Drug Response

LCC9 cells are resistant to endocrine therapies such as Tamoxifen and ICI 182,780 (ICI). This may be linked to the high Akt phosphorylation levels in these cell lines. Hence the effects of drug treatments following Akt RNAi transfection were investigated.

As previously observed in Chapter 3, MCF-7 cells require oestrogen to grow and are particularly sensitive to the anti-oestrogen ICI. Akt 1 and Akt 2 RNAi equally abrogated oestrogen response in these cells. Cell number remains unchanged in the presence of these Akt RNAis regardless of drug treatment as can be inferred by the unchanged OD values between day 3 and 5 (Figure 6.15).

Response to oestrogen in LCC1 cells is also lost after RNAi treatment but unlike in MCF-7 cells, all three Akt RNAis have this effect. In these cells, Akt 1 RNAi seems to yield the most pronounced responses whilst the effects of Akt 2 and AKt 3 RNAi are comparable (Figure 6.15). Growth of LCC9 cells is also reduced in the presence of Akt RNAi. Once more Akt 1 RNAi produces the most dramatic effects particularly at Day 3.

The growth inhibition mediated by ICI also appears to be altered following Akt RNAi treatment. In MCF-7 cells, ICI is still growth inhibitory following Akt 1 & 2 RNAi treatment (p-values 0.018 and 0.0001 respectively; E_2 vs ICI for each Akt RNAi).

Similarly, growth of LCC1 cells is also reduced in the presence of ICI and Akt RNAi (Akt 1, 2 & 3 RNAi p-value 0.0001 for all three). Resistant LCC9 cells remain unresponsive to ICI in the presence of Akt 2 and Akt 3 RNAi (p-values 0.702 & 0.645 respectively). Interestingly, Akt 1 RNAi renders these cells sensitive to ICI treatment (p-value 0.018). This is unexpected considering the previous observation that Akt 1 phosphorylation levels are low.

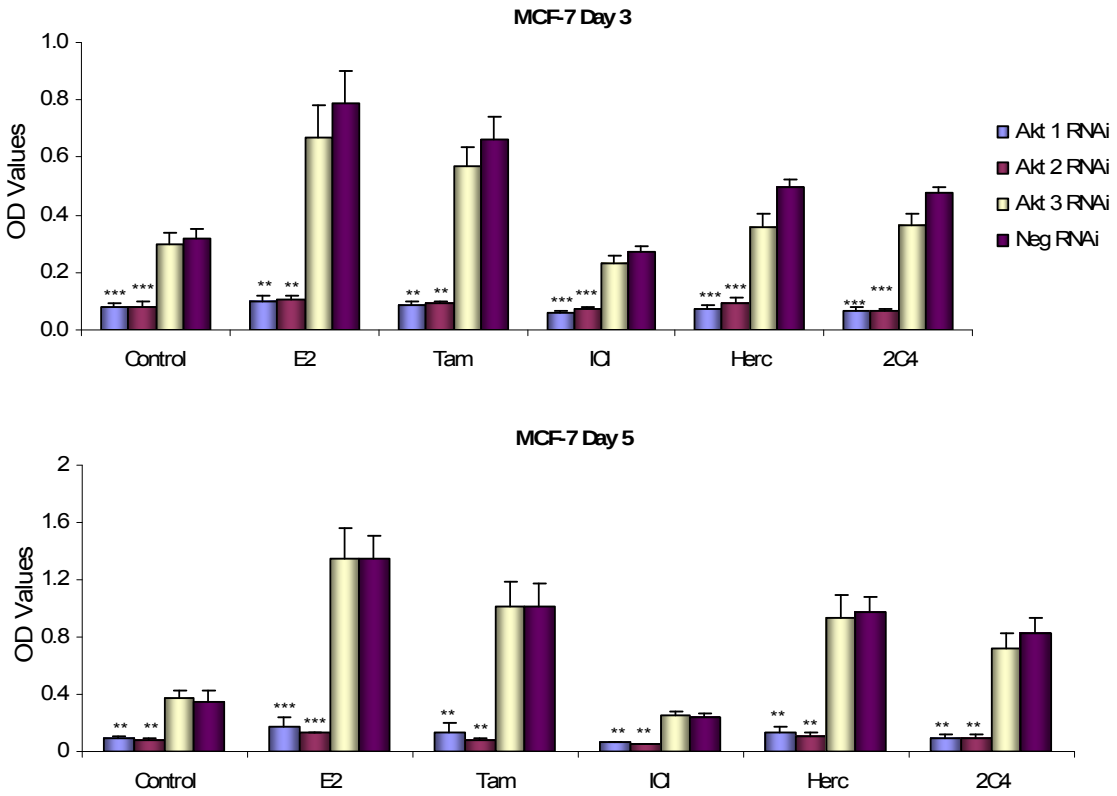


Figure 6.15 Effects of Akt RNAi on drug response in MCF-7 cells at day 3 and 5. Each column presents the mean of 6 OD values. Negative RNAi is used as a negative control. This ensures that any variations observed are due to the RNAi effect and not down to the effects of the transfection process. Error Bars=SD. Statistical analyses between each Akt RNAi treatment vs Negative RNAi (Student's unpaired t test **P<0.01; ***P<0.001).

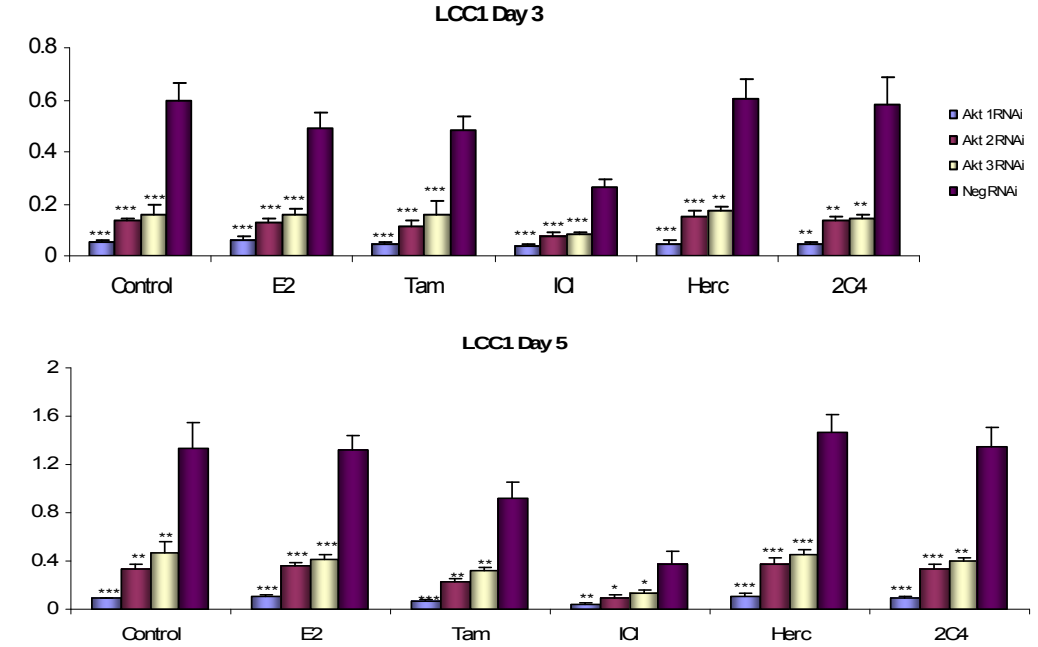


Figure 6.16 Effects of Akt RNAi on drug response in LCC1 cells at day 3 and 5. Each column presents the mean of 6 OD values. Negative RNAi is used as a negative control. This ensures that any variations observed are due to the RNAi effect and not down to the effects of the transfection process. Error Bars=SD. Statistical analyses between each Akt RNAi treatment vs Negative RNAi (Student's unpaired t test *P<0.05; **P<0.01; ***P<0.001).

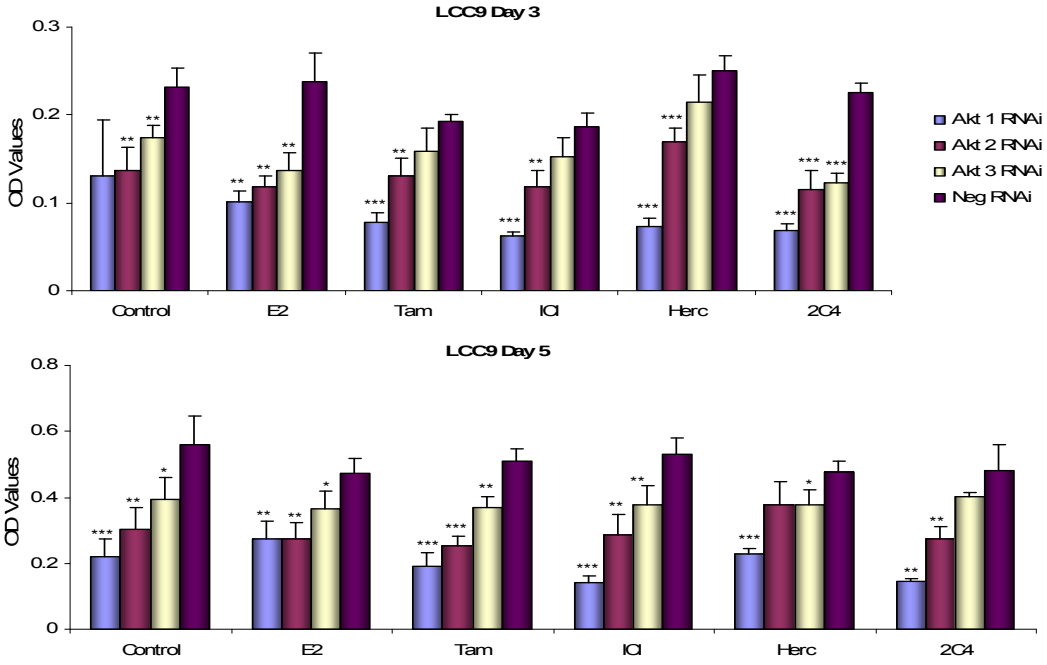


Figure 6.17 Effects of Akt RNAi on drug response in LCC9 cells at day 3 and 5. Each column presents the mean of 6 OD values. Negative RNAi is used as a negative control. This ensures that any variations observed are due to the RNAi effect and not down to the effects of the transfection process. Error Bars=SD. Statistical analyses between each Akt RNAi treatment vs Negative RNAi (Student's unpaired t test *P<0.05; **P<0.01; ***P<0.001).

6.5.4 Akt RNAi and ERα Activation

Taking into account the elevated Akt phosphorylation and consequent ERα activation (Ser167) in LCC1 and LCC9 cells it was important to establish the role of each Akt isoform in mediating these mechanisms.

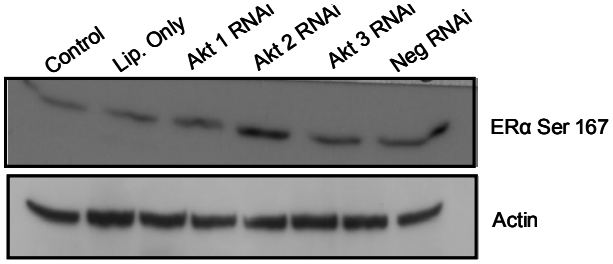


Figure 6.18 Western Blot analysis of Akt RNAi and the effects on ERα phosphorylation (Ser167) in LCC9 cells. All results shown are representative of three independent experiments. LCC1 western blot is not shown as these cells were found to be particularly sensitive to Akt RNAis and did not routinely yield enough protein to be able to run this experiment.

Neither Akt RNAi reduced Ser167 levels in LCC9 (Figure 6.18). This seems to suggest that none of the Akt isoforms are solely essential in mediating the high Ser167 levels observed in LCC1 and LCC9 cells. It is however important to consider that previous reports have suggested there may be a level of functional redundancy between these isoforms (Franke *et al*, 2000).

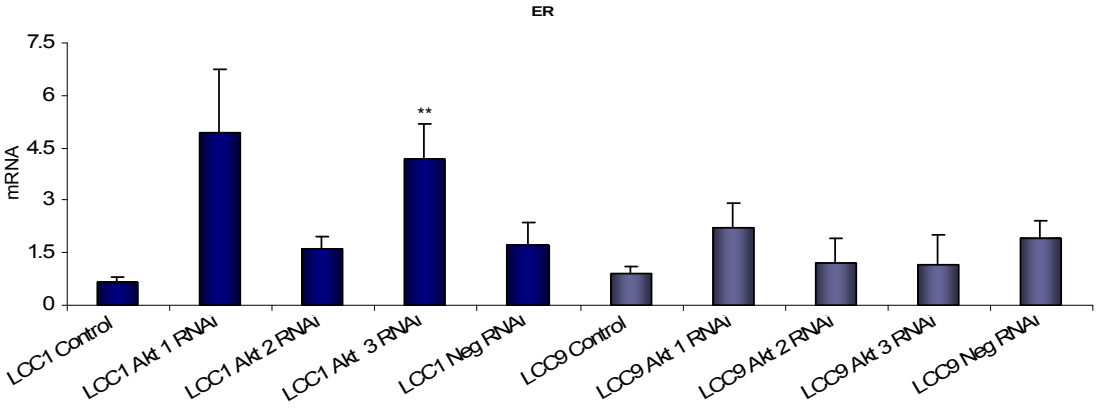


Figure 6.19 Effects of Akt RNAi on oestrogen receptor regulation. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars=SD. Statistical analyses between each Akt RNAi treatment vs Negative RNAi (Student's unpaired t test **P<0.01).

The effects of Akt RNAi on transcriptional regulation of ERα and pS2 were also investigated. ERα transcription in LCC1 appears to be higher following Akt RNAi transfection. This is particularly true when treated with Akt 1 and Akt 3 RNAi. Expression of ERα mRNA is not affected by Akt RNAi in LCC9 cells (Figure 6.19). pS2 regulation in LCC1 mirrors that of ERα as Akt 1 and Akt 3 RNAis also seem to induce an increase in mRNA levels (Figure 6.20). In LCC9 cells pS2 transcriptional regulation remains unchanged regardless of Akt RNAi treatment used (Figure 6.20). These results suggest the regulation of oestrogen responsive genes is not affected by reducing the expression of single Akt isoforms.

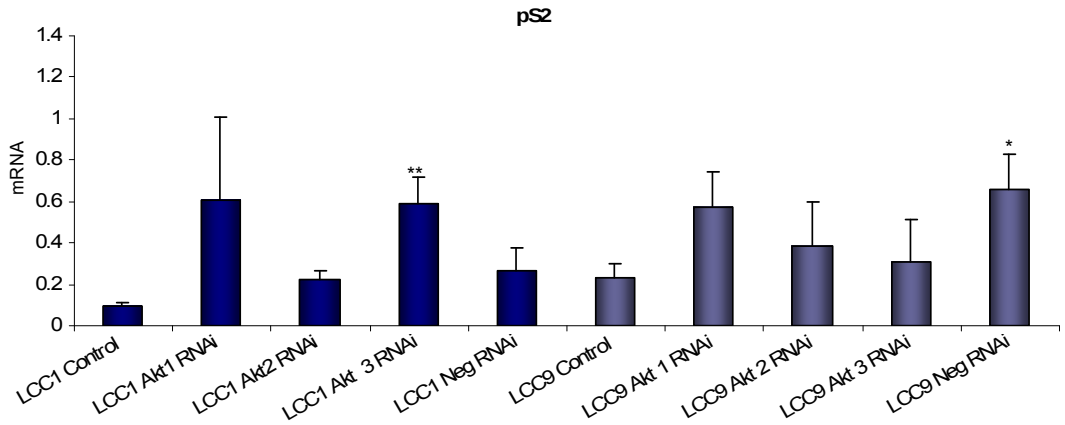


Figure 6.20 Effects of Akt RNAi on oestrogen regulated gene pS2. Each column presents mean of quadruplicate RT-PCR analysis for each sample relative to actin expression. Error Bars=SD. Statistical analyses between each Akt RNAi treatment vs Negative RNAi (Student's unpaired t test *P<0.05; **P<0.01).

6.6 Discussion

Akt mediates a number of cellular processes such as apoptosis, cell cycle, cellular proliferation and motility. It is therefore not surprising that this protein kinase is often deregulated in human disease including cancer (Nicholson *et al*, 2007). Constitutively activated Akt has been observed in a number of breast cancer cell lines (Clark *et al*, 2002¸Jordan *et al*, 2004 & Tokunaga *et al*, 2006) and is often associated with tamoxifen resistance in ER-positive breast cancer cells (Campbell *et al*, 2001; Kurokawa *et al*, 2003; Frogne *et al*, 2005). Elevated Akt phosphorylation is observed in LCC1 and LCC9 cells in comparison to the parental MCF-7 cell line suggesting Akt activation may be important in conferring anti-oestrogen resistance in these cells.

Regulation of Akt kinase activity is mediated by various growth factor-driven receptors. PI3K activation by such signalling cascades generates 3' phosphorylated phosphoinositides which specifically bind and activate Akt (Franke *et al*, 2003). Phosphorylated PI3K is elevated in LCC1 however this is certainly not the case in LCC9 cells. These results suggest that increased phosphorylation at the Ser473 is not dependent on PI3K activation. A number of other kinases have been proposed to be Ser473 kinases including mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) and integrin-linked kinase (ILK) (Shaw *et al*, 1998 & Delcommenne *et al*, 1998). Proteomic studies have revealed ILK depletion was associated with inhibition of Ser473 phosphorylation (McDonald *et al*, 2008). MAPKAP-K2 has also been shown to activate Akt and mutational analysis showed this kinase specifically targeted Ser473 and did not affect Thr308 phosphorylation (Alessi *et al*, 1996). The results in MCF-7, LCC1 and LCC9 cells suggest that the mechanism(s) regulating Ser473 phosphorylation may bypass PI3 kinase activation.

Mutations in the p110 catalytic subunit α of PI3K (*PI3KCA*) have been linked to tumour growth/invasion and have been implicated in breast cancer (Pérez-Tenorio *et al*, 2007). The most common mutations are located in exons 9 and 20 hence studies have focussed on these two loci. Mutational analysis revealed that the MCF-7 parental cell line harbours the same PI3KCA mutations as the LCC1 and LCC9 cells. There are conflicting reports regarding PIK3CA mutations in MCF-7 cells. Bachman et al (2005) report the MCF-7 cell line harbours the E542K mutation. Saal et al (2005) on the other hand show that these cells contain the E545K mutation only. The results presented in this thesis support Saal's findings. LCC1 and LCC9 cells are also positive for the E545K mutation but negative for the remaining mutations analysed, suggesting that PIK3CA mutations are not a contributing factor in acquired resistance in this cell model.

Akt is also negatively regulated by PTEN which removes the 3' phosphate group from the PI3K lipid product (McCubrey *et al*, 2008). Though PTEN mutations are relatively uncommon, PTEN protein loss due to promoter methylation or regulation at the RNA/protein level is observed in 30% of breast cancers (Singh *et al*, 1998; Garcia *et al*, 2004). PTEN knockdown in breast cancer cell lines induce PI3K and Akt activation (Miller *et al*, 2009) therefore it was important to determine whether loss of PTEN function mediates increased Akt phosphorylation in our cell line model. MCF-7, LCC1 and LCC9

cells express equal levels of basal and activated PTEN protein levels suggesting PTEN deregulation is not important in conferring resistance in these cells.

The components of the IGF system appear to contribute towards cellular proliferation, survival, angiogenesis and metastasis (Fagan *et al*, 2008). It is therefore not surprising that aberrant activation of IGF signalling has been implicated in breast cancer (Belfiore *et al*, 2008). Basal IGF receptor protein levels were equal in MCF-7, LCC1 and LCC9 cells despite reduced IGFR mRNA levels in LCC1 and LCC9 cells. Receptor phosphorylation is elevated in oestrogen independent LCC1 cells and endocrine resistant LCC9 cells which is likely to be the result of high ligand expression as IGFI and IGFII transcription is significantly higher in these cells. Increased IGFR activation is often observed in breast tumour tissue and is associated with reduced survival (Law *et* al., 2008). Moreover, IGFR expression appears to protect against damaging agents such as radiation and chemotherapeutic drugs by rescuing these cells from apoptosis. It has been proposed that this may be mediated by the downstream activation of PI3K and MAPK pathways (Jiang *et al*, 1999; Kuhn *et al*, 1999). Previous studies have also shown that increased signalling via the IGF receptor is often present in anti-oestrogen resistant cells (Campbell *et al*, 2001; Vivanco *et al*, 2002). In contrast, Frogne *et al* (2005) and Knowlden *et al* (2005) have shown reduced IGFR expression in anti-oestrogen resistant MCF-7 cell lines despite elevated Akt activation. These results suggest that increased PI3K/Akt activity is not always connected to elevated IGF signalling.

In line with the results presented in this chapter, IGFI and IGFII overexpression in MCF-7 cells have been shown to promote cell proliferation and tumour formation (Pacher *et al*, 2007) whilst elevated IGFI circulating levels are associated with increased breast cancer risk (Hankinson *et al*, 1998). IGFII overexpression, on the other hand, appears to inhibit apoptosis due to increased Akt activation (Moorehead *et al*, 2001). Collectively these results suggest that elevated IGF ligand expression may account for the elevated Akt phosphorylation and increased invading ability observed in LCC1 and LCC9 cells. Constitutive activation of Akt has also been shown to upregulate IGFR gene expression suggesting the Akt pathway is also involved in regulating IGFR expression (Tanno *et al*, 2001). According to these results, the increased IGFR activation observed in LCC1 and LCC9 cells may contribute towards increased phopshorylated Akt levels in these cells however Akt may also activate IGFR due to this feedback loop mechanism

In order to further determine the role of IGFR in MCF-7, LCC1 and LCC9 cells an IGFR kinase inhibitor, I-OMe AG538, was used. This compound binds to tyrosines 1158 and 1162 which undergo autophosphorylation during IGFR activation. Binding of I-OMe AG538 inhibits autophosphorylation and consequent IGFR inhibition (Blum *et al*, 2000). The I-OMe AG538 compound did not reduce cell growth nor did it reverse tamoxifen and ICI 182,780 resistance in LCC9 cells. Furthermore, cell growth was only reduced in MCF-7 cells. This is in contrast to results reported by Knowlden *et al* (2005). Their studies show IGFR inhibitors or IGFII neutralizing antibodies significantly reduced growth of tamoxifen-resistant cells. Furthermore, crosstalk between IGFR and EGFR/HER2 may also been important as reported by Nahta *et al* (2005) and Camirand *et al* (2005). Both studies show IGFR blocking may or may not individually inhibit cellular growth but IGFR inhibition helps overcome herceptin resistance and acts synergistically with gefitinib (EGFR inhibitor). IGFR inhibition did not reduce cellular proliferation in LCC1 and LCC9 cells. This could be the result of the IGFR inhibitor not actually inhibiting its target sufficiently. On the other hand, this could be a reflection of not high enough inhibitor concentration or unspecific inhibition leading to insufficient IGF repression. Finally it could be that the inhibition of IGFR alone is not sufficient and other membrane receptors may be important in this process.

Akt has been postulated to phosphorylate various proteins thereby mediating several signalling pathways and cellular processes (Hay, 2005). Akt is consequently regarded as a regulatory switch. Downstream targets include BAD and Forkhead transcription factors which regulate the apoptotic pathway; **g**lycogen **s**ynthase **k**inase-3β (GSK-3β) a promoter of cell growth and NF-κB which induces gene expression (McCubrey *et al*, 2008; Hanada *et al*, 2004; Toker *et al*, 2006; Fry, 2001). mTOR is also a downstream target of the Akt pathway. This protein is important in regulating cell cycle entry and initiation of protein synthesis in response to the adequate signals (Fingar *et al*, 2004). mTOR activation is elevated in LCC1 and LCC9 cells, however the basal protein levels are also high. This makes it difficult to determine whether increase mTOR phosphorylation is a result of elevated Akt signalling or simply a consequence of high protein expression. This could be tested by checking mTOR levels after Akt siRNA which would further help establish the role of Akt. Akt-mediated endocrine resistance has been shown to be reversed in the presence of mTOR inhibitors highlighting the role of mTOR (Beeram *et al*, 2007; DeGraffenried *et al*, 2004). mTOR activation has also been shown to be increased

progressively as normal breast epithelium develops into abnormal hyperplasia and tumour invasion (Zhou *et al*, 2004) . Reports also suggest mTOR is associated with more severe prognosis in breast cancer (Klos *et al*, 2006).

mTOR phosphorylates members of the protein synthesis machinery such as **p**70 **r**ibosomal **s**ix **k**inase (p70S6K) and **e**ukaryotic **i**nitiation **f**actor (eIF-4E)-binding protein (Yamnik *et al*, 2009; Wendel *et al*, 2004). p70S6K phosphorylates S6, the 40S ribosomal protein consequently initiating protein translation. In fact, LCC1 and LCC9 cells express high levels of phosphorylated S6 ribosomal protein suggesting elevated mTOR activation induces mRNA translation and protein synthesis in endocrine resistant cell lines.

Serine 167 is one of the major phosphorylation sites of ERα and Akt has since been identified as one of the kinases mediating ERα phosphorylation at this site (Campbell *et al*, 2001). Elevated Ser167 phosphorylation has been reported to be a consequence of increased Akt activity in acquired tamoxifen resistance (Shou *et al*, 2004; Nicholson *et al*, 2004). Furthermore, Ser167 phosphorylation appears to play a role in reversing the inhibitory effects of oestrogen deprivation (Staka *et al*, 2005). Phosphorylation at this serine site is markedly higher in LCC1 and LCC9 cells than in parental MCF-7 cells. This may play an important part in rendering these cells oestrogen independent and anti-oestrogen resistant due to ligand-independent ER activation.

These results indicate increased Akt activation in LCC1 and LCC9 cells may confer endocrine resistance firstly by promoting protein synthesis and secondly by inducing oestrogen independent ER phosphorylation.

Previous studies have shown that different Akt isoform expression is observed in endocrine resistance. Akt 1 and Akt 2 expression is unaltered in MCF-7, LCC1 and LCC9. This is an interestingly result considering these two Akt isoforms are ubiquitously expressed in mouse tissue whilst Akt 3 expression appears to be more restricted (Hanada *et al*, 2003). However, Akt 3 protein expression is markedly elevated in oestrogen independent LCC1 and anti-oestrogen LCC9 cells, which seems to be a result of increased transcriptional rate of this gene. The Akt 3 isoform has been less studied yet evidence suggests that Akt 3 expression is associated with hormone-independence, ER-negative and more advanced breast cancers (Nakatani *et al*, 1999). Analysis of normal versus tumour tissue suggested Akt isoform expression is not altered in tumorigenesis (Zinda *et al*, 2001). On the other hand, constitutively activated Akt3 expression in MCF-7 cells promoted

oestrogen independent tumour growth (Faridi *et al*, 2003). This data supports the results presented here and suggest the Akt 3 isoform may be important in tumorigenesis of these breast cancer cell lines.

Isoform activation is also altered in these cell line models. Immunoprecipitation experiments suggest Akt 3 isoform is the preferred phosphorylated target in MCF-7, LCC1 and LCC9 cells. This is surprising considering basal Akt 3 levels are apparently very low in parental MCF-7 cells. Despite previous reports suggesting Akt 3 activation may play a part in conferring endocrine resistance (Faridi *et al*, 2003), Akt 3 phosphorylation is present in endocrine sensitive MCF-7 cells as well as in the fully resistant LCC9 cells. These results indicate that Akt 3 activation may be important in these cells however, it does not appear to mediate endocrine resistance. Interestingly, Akt 2 phosphorylation is elevated in LCC1 and LCC9 cells. Activation of the Akt 2 isoform has been previously shown to offer protection against docetaxel induced apoptosis in breast cancer cells. Moreover, increased Akt 2 kinase activity confers survival advantage (Xing *et al*, 2008) and appears to be present in approximately 40% of breast cancer cases (Sun *et al*, 2001). These results suggest that Akt 2 is important in breast cancer and that increased activation observed in LCC1 and LCC9 cells may be important in conferring resistance.

RNAi studies revealed Akt 1 and Akt 3 RNAis reduce cellular proliferation in all three cell lines suggesting that these isoforms are important despite differential phosphorylation. Oestrogenic stimulation is abrogated by Akt 1 and Akt 2 in MCF-7 cells indicating they may play a part in mediating oestrogenic action. Akt RNAi treatment reduces cellular proliferation in LCC9 cells but this was not exarcebated by tamoxifen or ICI 182,780 treatment. Hence no isolated Akt isoform RNAi treatment appears to restore anti-oestrogen sensitivity. Pancholi *et al* (2008) have recently reported that proliferation of a tamoxifen resistant MCF-7 cell line is significantly reduced after Akt inhibition mirroring the results obtained in LCC1 and LCC9 cells. Akt is known to promote cell survival by inhibiting pro-apopotic pathways hence it has been proposed that Akt inhibition reduces proliferation as a result of increased apoptosis. Mouse studies revealed that the knockout of each individual isoform generate different phenotypes indicating they regulate distinct cellular mechanism (Chen *et al*, 2001; Cho *et al*, 2001; Easton *et al*, 2005; Tschopp *et al*, 2005). Moreover, Akt RNAi studies suggest isoforms may have opposing roles since Akt 1 is described as an inhibitor of cell migration whilst Akt 2 promotes cellular motility and invasion (Irie *et al*, 2005). A study by Maroulakou *et al* (2007) in mice also supports these

findings as they report ablation of Akt 1 and Akt 2 promoted and inhibited tumour growth, respectively. Akt 3 ablation appeared to have limited effects on cellular proliferation (Maroulakou *et* al, 2007). The specific role of individual Akt isoforms in endocrine resistance is yet to be fully determined.

Previous studies have shown that Akt RNAi alone did not affect ERα phosphorylation at the Ser167 residue (Pancholi *et al*, 2008) as observed in our results. However, combination of Akt RNAi and MAPK RNAi did reduce phosphorylation levels (Pancholi *et al*, 2008). This indicates that Akt may not be the only protein mediating elevated ERα activation in LCC1 and LCC9

The transcription of oestrogen responsive genes is often elevated in endocrine resistant cells. In fact, previous work by our group has shown pS2 and ERα basal mRNA expression is elevated in LCC1 and LCC9 cells (Kuske *et al*, 2006). Akt RNAi did not affect ER and pS2 transcriptional rates suggesting Akt is not responsible for the elevated transcriptional rates of oestrogen responsive genes often observed in endocrine resistance. An overview of the PI3K/Akt pathway is shown in Figure 6.21. The proteins found to be overexpressed in LCC1 and LCC9 cells are highlighted in the diagram

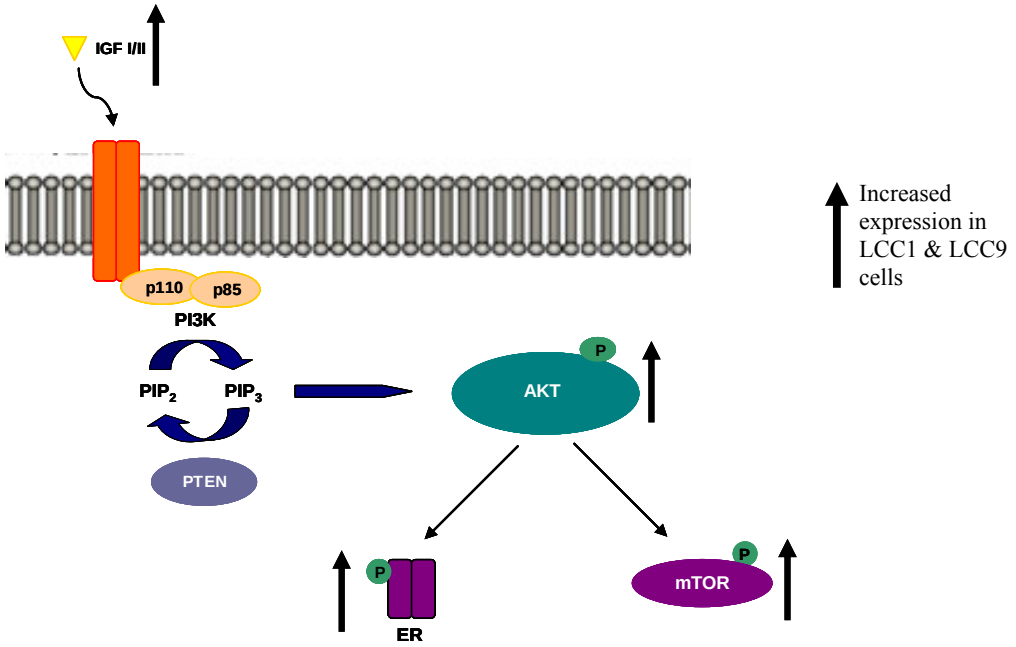


Figure 6.21 PI3K/Akt pathway overview. A summary of the proteins analysed in this study. The block arrows depict the members of the pathway whose expression is elevated in oestrogen independent LCC1 and endocrine resistant LCC9 cells.

Chapter 7

Discussion

The advent of endocrine resistance in breast cancer remains one of the major challenges in the treatment of this condition. Various mechanisms have been implicated in endocrine resistance however how the complex regulatory networks may interact has yet to be fully established. The thorough understanding of crosstalk between signalling cascades and their effect on cellular processes such as proliferation, cell cycle and apoptosis would facilitate the development of combinatorial therapies targeting resistance (Arlt *et al*, 2006).

A three-stage MCF-7 cell-based model for oestrogen independence and endocrine resistance was used in this study to determine which intracellular signalling pathways may be important in conferring resistance to anti-oestrogenic drugs. The LCC1 cell line, which was derived *in vivo* from MCF-7 cells, appears to mimic clinical progression from early stage to a more aggressive phenotype. LCC1 cells are oestrogen independent but retain sensitivity to endocrine treatments suggesting this phenotype arises independently of endocrine resistance (Leonessa *et al*, 1992; Brünner *et al*, 1993). On the other hand, LCC9 cells were derived *in vitro* following stepwise selection in the presence of the pure anti-oestrogen ICI 182,780. This cell line is fully resistant to ICI and tamoxifen despite never being exposed to the latter (Brünner *et al*, 1997). During the acquisition of endocrine resistance, ER positive breast cancer cells often progress in a stepwise manner from full oestrogen dependence to an oestrogen-sensitive, but no longer dependent phenotype, culminating in complete endocrine resistance (Clarke *et al*, 2001; Clarke *et al*, 2003). The MCF-7-base cell line model use in this study emulates this process observed in a clinical setting.

A number of studies using these cells lines have suggested that endocrine resistance does not appear to be associated with detectable copy number alterations suggesting oestrogen independence and endocrine resistance may result from changes in gene expression and/or protein activation and degradation (Brünner *et al*, 1993; Johnson *et al*, 2008). As shown here in Chapter 3 and by Kuske *et al* (2006), ERα expression is markedly elevated in LCC1 cell lines but not in the resistant LCC9 cells. Higher ERα protein expression is linked to increased cellular proliferation and to promoter occupancy and consequent gene activation in the absence of ligand binding (Dowsett *et al*, 2005; Fowler *et al*, 2004). However, while high ERα levels may be associated with enhanced oestrogen response, it may also render certain tumours resistant to endocrine therapies (Kuske *et al*, 2006). Loss

of ERα protein expression or function is found to confer *de novo* resistance to anti-oestrogenic therapies and is present in 30 to 40% of breast cancer patients (Ma *et al*, 2009). The LCC1 cells shares similar characteristics to the long-term oestrogen deprivation (LTED) models generated following culturing in low-oestrogen conditions. These LTED cells are considered oestrogen hypersensitive due to their ability to respond to oestrogen levels 2 to 3-log lower that the levels required to stimulate wild-type cells (Yue *et al*, 2003; Martin *et al*, 2005). These cell lines, like LCC1 cells, are characterised by higher ERα protein expression and the ability to grow in low oestrogen conditions (Yue *et al*, 2002; Martin *et al*, 2005; Santen *et al*, 2005). Despite this, Kuske *et al* (2006) show that unlike the LTED models, LCC1 cells fail to respond to low levels of exogenous oestrogen.

The higher ERα levels observed in LCC1 cells are probably a result of routine culture in low oestrogen conditions. Oestrogen reduces ERα in MCF-7 cells and its derivatives cell lines indicating the existence of protein turnover and proteasomal degradation of ERα (Nawaz *et al*, 1999). siRNA removal of ERα inhibited the growth of both LCC1 and LCC9 cells indicating a dependency on the receptor (Kuske *et al*, 2006).

The transcription of oestrogen regulated genes, such as trefoil factor 1 (TFF1)/ pS2, is elevated in LCC1 and LCC9 cells under basal conditions suggesting ligand-independent ERα function is an important mechanism at play in this cell line model. The oestrogen receptor is primarily activated by the presence of its ligand leading to protein phosphorylation (Reviewed in Lannigan *et al*, 2003). However, ligand-independent activation has also been extensively documented and has been shown to play a part in bypassing the tumour's requirement for oestrogen. Signalling pathways such as MEK/ERK and Akt are able to directly phosphorylate ERα at serines 118 and 167, respectively (Bunone *et al*, 1996; Martin *et al*, 2000).

Receptor phosphorylation at the serine 167 residue is markedly increased in oestrogen-independent LCC1 and endocrine resistant LCC9 cells in comparison to the parental MCF-7 cell line. Activation of Akt, which directly phosphorylates this residue, is also elevated in these cell lines suggesting these two events may be connected. Therefore, this pathway became one of the focuses of this study. However, unlike serine 167 residue and Akt, serine 118 phosphorylation and MEK/ERK activation are not increased in LCC1 and LCC9 cell lines suggesting this pathway is not important in conferring endocrine resistance in this cell line model. Work by Gratton *et al* (2001) and others have reported the existence of crosstalk between Akt and MEK/ERK pathways. They have observed that the activation

of the PI3K/Akt pathway led to the inhibition of MEK thereby reducing stress kinase activation and protecting cells from apoptosis (Gratton *et al*, 2001; Park *et al*, 2002; Zimmermann *et al*, 1999). This does not appear to be important in the LCC cell line models as MEK/ERK activation remains unchanged despite Akt phosphorylation being markedly elevated.

The Akt kinase has become central in many cancer studies because it mediates a number of distinct cellular processes such as proliferation, apoptosis and cellular motility (Pecorari *et al*, 2009). Apoptosis levels after treatment with anti-oestrogens and anti-HER2 therapies, as measured by PARP cleavage, are reduced in endocrine resistant LCC9 cells in comparison to oestrogen independent LCC1 cells. This is in agreement with previous studies by Riggins *et al* (2005) which report LCC9 cells have reduced levels of ICI 182,780 mediated apoptosis. Various studies have suggested that apoptosis bypass in these cell lines may be regulated by NFκB. The expression of this transcription factor is elevated in LCC9 cells and its regulation is also altered as oestrogen withdrawal and ICI 182,780 treatment no longer inhibit NFκB expression (Gu *et al*, 2002; Pratt *et al*, 2003). Increased Akt phosphorylation is present in both LCC1 and LCC9 cell lines suggesting that perhaps there is a secondary mechanism further increasing apoptosis resistance in the fully endocrine resistant LCC9 cells.

Previous reports have also suggested that actin is a substrate of Akt and that this kinase influences the organization of the actin cytoskeleton (Vandermoere *et al*, 2007). The involvement of the PI3K/Akt pathway in the control of cell migration was suggested following reports that the activation of this pathway led to anchorage-independent growth and metastasis of liver and thyroid carcinoma cells (Nakanishi *et al*, 2002; Kim *et al*, 2005). In addition, Akt was found to down-regulate RhoB, a protein known to suppress migration and metastasis (Jiang *et al*, 2004). Migration in LCC1 and LCC9 cells remained either unchanged or actually reduced in comparison to the parental MCF-7 cell line. This is in agreement with the phenotypic data which indicates that MCF-7 cells tend to grow as a monolayer spreading across the surface area available whilst LCC1 and LCC9 cells preferentially grow in “clumps”. On the other hand, the invading ability of the latter two cell lines was significantly increased. This has been previously reported to be the case in the LCC1 cell line (Castro *et al*, 2005).

As well as promoting cell invasion and survival, Akt upregulation inactivates negative cell cycle regulators such as p21 and p27 thereby leading to cell cycle progression and cell

growth (Zhou *et al*, 2001; Liang *et al*, 2002; Shin *et al*, 2002). Both LCC1 and LCC9 cells have a higher percentage of cells found at S-phase under basal conditions. This is in contrast to the MCF-7 parental cell line which requires oestrogen to progress through the cell cycle.

The activation of mTOR, a "downstream" effector of Akt, was also found to be elevated in LCC1 and LCC9 cell lines. mTOR is important to for the oncogenic transformation induced by the PI3K/Akt pathway. Activation of mTOR results in the phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 and the ribosomal p70 S6 kinase which activate protein translation (Albert *et al*, 2006). Phosphorylation of the S6 kinase was found to be increased in LCC1 and LCC9 cells presumably as a result of elevated mTOR activation. This may be a reflection of increased protein translation rates in LCC1 and LCC9 cells which could be in part responsible for increased protein levels.

Together, this data indicates that Akt activates and regulates a number of different cellular processes which combined may confer oestrogen independence and endocrine resistance. These include alterations in apoptosis, cell cycle and invading ability as well as increase in translation. It is very likely that the exact role of Akt is different in LCC1 and LCC9 cells despite both these cell lines overexpressing this kinase. It is important to remember that LCC1 are oestrogen independent but remain sensitive to anti-oestrogen therapies unlike LCC9 cells. Moreover, whilst cell cycle progression is not oestrogen-dependent in LCC1 and LCC9 cells, ICI 182,780 still induces a G_0/G_1 arrest in LCC1 cells only suggesting that the cell cycle in these cells continues to, at some level, be regulated by oestrogen signalling.

Akt activation in breast cancer is linked to resistance to chemo-, hormone- and radiotherapy-induced apoptosis, predicts for a more aggressive behaviour and correlates with reduced overall survival (Bellacosa *et al*, 2005; Sutherland *et al*, 2005). PI3K activation generates second messenger PtdIns-3,4,5-P_3 which bind to Akt and recruit this kinase to cell membrane. On the other hand, Akt is negatively regulated by PTEN. Loss of PTEN function and activating mutations in the PI3K gene are frequently observed in breast cancer tumours. Deregulation of these pathways may be responsible for elevated Akt activation. However, this was not found to be the case in these cell lines as PTEN expression was unchanged and no PI3K mutations were detected suggesting these

mechanisms are not responsible for the increased Akt phosphorylation levels observed in LCC1 and LCC9 cells.

On the other hand, IGF receptor phosphorylation is increased in LCC cells. The IGF signalling comprises of a complex interacting network of receptors and ligands which activate multiple pathways such as the PI3K/Akt and MAPK pathways (Reviewed in Samani *et al*, 2007). LCC1 and LCC9 cells were found to markedly overexpress the IGF ligands, IGFI and IGFII. These ligands have been identified as potent mitogens important in maintaining the transformed phenotype (Hollier *et al*, 2008). Ligand overexpresssion will induce increased receptor phosphorylation which in turn activates downstream signalling pathways such as Akt. Given that PTEN and PI3K functions are not altered in these cell lines, elevated IGF receptor phosphorylation was considered the most likely Akt activator in LCC1 and LCC9 cells. The IGF system may also be crucial in conferring resistance via distinct pathways and not necessarily requiring Akt. For example, the expression of IGFII has been shown to override the need for oestrogen in MCF-7 cancer cells with the ligand being the limiting factor rather than the receptor (Stephen *et al*, 2001). Furthermore, it has also been established that IGFs directly regulate the activation of the oestrogen receptor (Fagan *et al*, 2008).

Despite these results, I-OMe AG538, an IGFR kinase inhibitor which blocks receptor autophosphorylation, did not restore endocrine sensitivity and oestrogen dependence in LCC9 and LCC1 cell lines, respectively. This is in contrast to previous studies by Knowlden *et al* (2005) where they report IGFR inhibitors reduced the growth of tamoxifen-resistant cells. Conversely, other groups report that IGFR inhibition alone may not be sufficient to illicit a reduction in cellular growth (Jones *et al*, 2004; Camirand *et al*, 2005; Jones *et al*, 2006). Elevated IGFR signalling is often observed in cells lines resistant to anti-EGFR therapies and IGF activation is essential in EGFR-mediated cellular proliferation (Chakravarti *et al*, 2002; Stull *et al*, 2002). These studies highlight the interaction between the two pathways therefore the combination of IGF and EGF targeted therapies may be more efficient in inhibiting growth. Furthermore, it has been reported that IGFR interacts and activates HER2 (Balana *et al*, 2001). As observed in EGFR signalling, overexpression of IGFR was associated with resistance to anti-HER2 therapies (Lu *et al*, 2001). A shift between IGFR, EGFR and HER2 signalling may be responsible for the absence of response to IGFR blocking and targeting the three receptors may be necessary for complete growth inhibition.

The aberrant activation of cell membrane receptors has also been extensively linked to the increased signalling through downstream pathways including proliferation, survival and metastasis (Reviewed in Jin *et al*, 2008) The EGF family comprising EGFR, HER2, HER3 and HER4 is of particular interest due to its role in malignant transformation and cancer progression (Hynes *et al*, 2005). EGFR, HER2 and HER3 are frequently overexpressed in breast cancer and are associated with a more aggressive behaviour, poor prognosis and reduced overall survival (Klijn *et al*, 1992; Owens *et al*, 2004). The protein expression of EGFR, HER2 and HER3 is not elevated in oestrogen independent LCC1 and endocrine resistant LCC9 cells.

These results suggest that the role of these receptors may be limited in this cell line model. However, oestrogen regulation of HER2 expression was found to be altered in resistant LCC9 cells. Whilst in MCF-7 and LCC1 cells, oestrogen reduces HER2 expression at the transcriptional and protein levels, this is not observed in LCC9 cells. Oestrogen mediated HER2 downregulation has been a known mechanism for quite some time (Dati *et al*, 1990; Read *et al*, 1990; Russell *et al*, 1992) and the results shown here prove this is not confined to the HER2 receptor and also affects the expression of HER3 and HER4 expression. Moreover, the results in Chapter 4 show that oestrogen-mediated HER3/HER4 repression is lost in LCC9 cells. The regulatory mechanism behind oestrogen transcriptional inhibition was shown here and by others to be mediated by the p160 coactivator family (Newman *et al*, 2000; Hurtado *et al*, 2009). The SRC-1 and AIB1 coactivators were shown to be transcriptional activators of HER2 which are preferentially recruited to the oestrogen receptor following oestrogenic stimulation. Transcriptional regulation of HER2 in LCC9 cells is altered despite remaining responsive to action of SRC-1 and AIB1, as evidenced by the double knockout experiments. ChIP experiments would also help reveal which proteins bind and activate the enhancer region of the HER2 promoter. This would be likely to change following oestrogen treatment and it would be interesting to establish whether this is altered in the advent of endocrine resistance.

The link studied here between p160 coactivators and the transcriptional regulation of the *HER2* gene is likely to also be applicable to the other membrane receptors HER3 and HER4. Furthermore, altered transcriptional regulation of other genes including other cell membrane receptors despite no obvious protein overexpression may be an important feature of resistance. It would be interesting to establish whether this is the case for other receptors such as the IGF receptor. In addition, a number of proteins have now been

identified as inhibitors of HER2 transcription (e.g. PAX2; FOXP3; PEA3). It would be interesting to determine whether expression of these inhibitor proteins is at all altered in oestrogen-independent LCC1 and endocrine resistant LCC9 cells.

In conclusion, oestrogen independence and endocrine resistance in LCC1 and LCC9 cells respectively may be intrinsically connected to increased/deregulated signalling via membrane receptors such as IGFR, HER2 and HER3. These cell membrane receptor activate a number of downstream signalling pathways such as Akt. As a result various cellular processes are affected: cell survival is increased due to the ability to bypass apoptosis, cell cycle arrest is overcome and invading ability is increased. These alterations combined are likely to contribute towards an oestrogen independence phenotype and ultimately endocrine resistance.

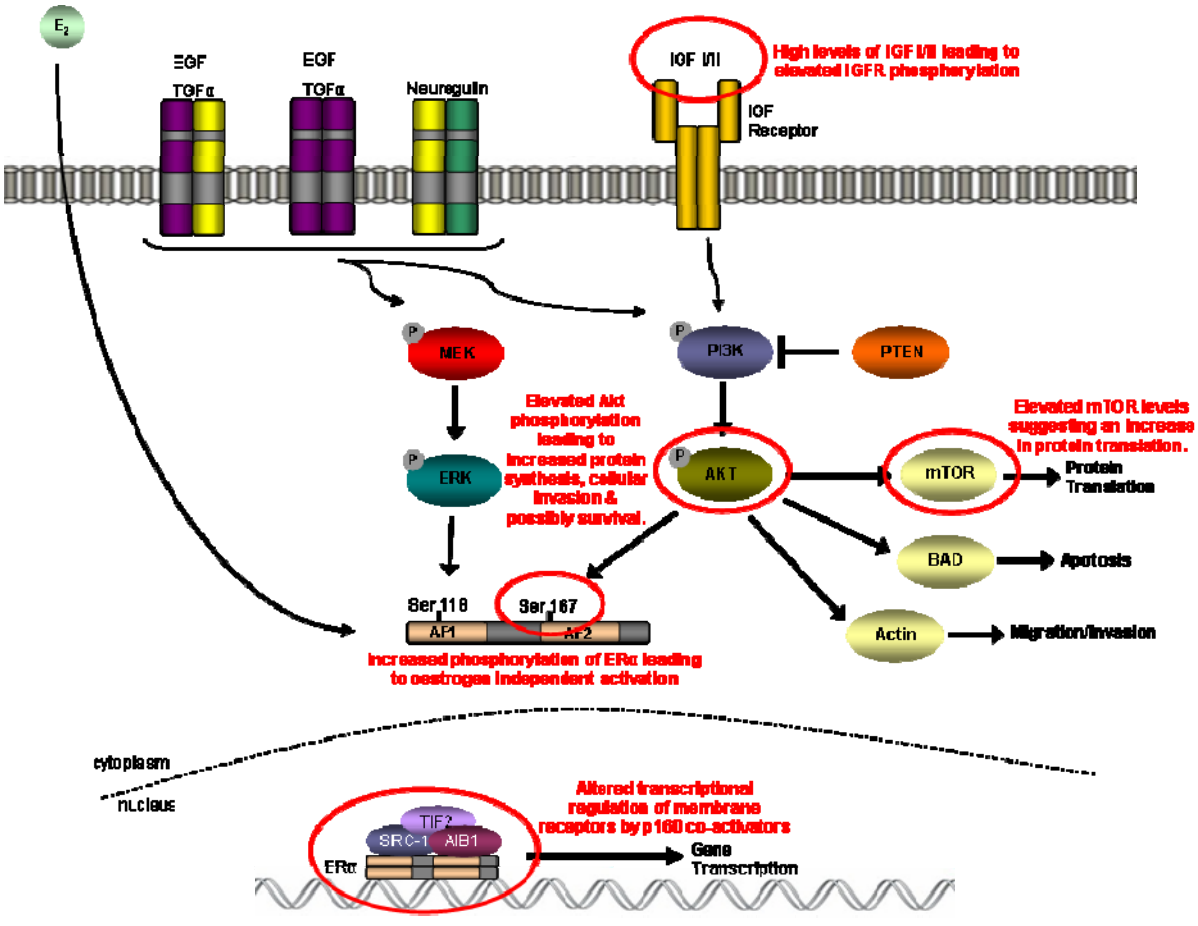


Figure 7.1 Overview of pathways and mechanisms found to be altered in endocrine resistant LCC9 cells. The areas which were found to be altered are highlighted in red with a brief description of the major findings.

Chapter 8

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Conclusions

The development of endocrine resistance is one of the major challenges in current therapeutic approaches in breast cancer treatment. Several mechanisms have been implicated in breast cancer endocrine resistance such as HER2 upregulation. This has been particularly important as it gave rise to new drug therapies (e.g. Herceptin) specifically targeting this protein.

In this study, an MCF-7-based cell line model emulating the clinical development of endocrine resistance was used to establish which intracellular pathways may be important in conferring resistance to anti-oestrogens. Transcriptional regulation of HER2, 3 and 4 is altered in oestrogen independent LCC1 and endocrine resistance LCC9 cells in comparison to the parental MCF-7 cells. Expression of these receptors is downregulated in the presence of oestrogen which is not observed in LCC1 and LCC9 cells. Therefore, despite no detectable overexpression of these receptors, HER-driven signalling regulation is altered in LCC1 and LCC9 cells. The p160 co-activator family (SRC-1, TIF2 and AIB1) were shown to be particularly important as they bind and activate transcription. In the parental MCF-7 cell line, the SRC-1 co-activator appeared to be the main activator of HER2 transcription as confirmed by the ability of SRC-1 RNAi to mimic oestrogen in downregulating HER2 expression. In contrast, SRC-1 and AIB1 are both required in HER-transcription activation in LCC1 and LCC9 cell lines. This shift ensures that in the presence of oestrogen HER2 transcription is not downregulated as there are two available co-activators instead of one. Furthermore, herceptin and 2C4 which target HER2, are not as effective in promoting apoptosis in LCC9 cells suggesting by bypassing the HER2 regulatory mechanisms, these cells are able to reduce apoptosis and increase cellular survival.

In addition to altered HER regulation, Akt activation is markedly elevated in LCC1 and LCC9 cells. This appears to result from increased IGF receptor signalling as both cell lines express high levels of IGFR ligands (IGFI and IGFII). Increased Akt activation mediates elevated mTOR phosphorylation thereby promoting translation and protein synthesis. Furthermore, Akt also induces ligand-independent ERα activation by directly phosphorylating the receptor at the Ser167 residue. This may be crucial in overcoming

the effects of anti-oestrogen therapies and consequently conferring resistance. Increased Akt 2 phosphorylation is associated with increased oestrogen independence and endocrine resistance since it is markedly elevated in LCC9 cells but only to a small extent in LCC1 cells.
LCC9 and LCC1 cells also show enhanced invasion ability in comparison to the parental MCF-7 cells consistent with previous studies have shown (Vandermoere *et al*, 2007) and this may be a result of increased Akt phosphorylation.

Together these results indicate that oestrogen independence and endocrine resistance in these cells may be mediated by increased HER receptor signalling and elevated Akt activation. These two pathways may well be connected as these receptors are upstream activators of pathways such as Akt hence conferring survival advantage to the cells by reducing the number of cells undergoing apoptosis and increasing their invasion ability ability. Finally, increased activity of these pathways ensures ligand-independent activation of ERα and signalling cascades activated via the non-genomic pathway thereby conferring resistance to these cell lines. These results further suggest that efficient breast cancer treatments may require different drugs to target distinct parts of the signalling cascades.

Future Studies

A number of further studies would be very valuable and informative to perform in order to complete and extend the experiments in this thesis:

1. As shown in Chapter 4, oestrogen downregulates HER2, HER3 and HER4. It would be interesting to establish whether this is the case for other receptors, such as the IGF receptor.
2. A number of proteins have now been identified as inhibitors of HER2 transcription (e.g. PAX2; FOXP3; PEA3). It would be interesting to determine whether expression of these inhibitor proteins is at all altered in oestrogen-independent LCC1 and endocrine resistant LCC9 cells.
3. ChIP experiments would also help reveal which proteins bind and activate the enhancer region of the HER2 promoter. This would be likely to change following oestrogen treatment and it would be interesting to establish whether this is altered in the advent of endocrine resistance.
4. Akt RNAi and immunoprecipitation studies indicate that increased Akt 2 phosphorylation is associated with endocrine resistance however all three isoforms are important in mediating cellular growth and oestrogen response. In order to further determine their individual roles, it would be informative to perform double Akt isoform knockouts.
5. Further establish how Akt RNAi affects cellular processes such as apoptosis and invasion which have shown to be altered in the resistant cell line model.
6. Investigate IGF receptor signalling and the activation of the downstream Akt pathway. As observed in Chapter 6, the expression of the IGFI and IGFII ligands is elevated in LCC1 and LCC9 cells hence activation of IGFR may be responsible for elevated Akt phosphorylation. Using efficient IGFR inhibitors would help clarify its role in oestrogen independence and endocrine resistance.